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Patent Application Transmittal
(only for new nonprovisional applications under 37 C.F.R. 1.53(b))
Correspondence Address:
FROMMER LAWRENCE & HAUG LLP
745 FIFTH AVENUE
NEW YORK, NEW YORK 10151
TEL: (212) 588-0800
FAX: (212) 588-0500

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Attorney Docket No.: 454313-2200.1

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Sir:

With reference to the filing in the United States Patent and Trademark Office of an application for patent in the name(s) of:

JEAN-CHRISOPHE AUDONNET and PHILIPPE BAUDU

entitled:

**CANINE HERPESVIRUS BASED RECOMBINANT LIVE VACCINE, IN PARTICULAR
AGAINST CANINE DISTEMPOR, RABIES OR THE PARAINFLUENZA 2 VIRUS**

X Continuing Application

 Continuation X Divisional Continuation-in-Part (CIP)
of prior application serial no.09/213,053, filed December 16, 1998, which is a
continuation of PCT/FR97/0115, filed June 23, 1997.

[Note: If priority under 35 U.S.C. 120 involves a series of respectively copending applications, then in this amendment identify each and its relationship to its immediate predecessor.]

X The prior application is assigned of record to MERIAL.

 This is an application of a small entity under 37 CFR 1.9(f) and the amounts shown in parentheses below have been employed in calculating the fee:

- ☐ Small Entity Verified Statement(s) is (are) enclosed.
☐ Small Entity Verified Statement(s) filed in prior application, status still proper and desired

The following are enclosed:

- ☒ Specification (23 pages)
☒ 26 Sheet(s) of Drawings (Figs 1, 1 continued, 1 continued, 2-15))
☒ 16 Claim(s) (pages 24-25)

☐ This application contains a multiple dependent claim (see Preliminary Amendment herewith)

☒ Our check for \$690.00, calculated on the basis of the claims as amended by any enclosed preliminary amendment as follows:

Basic Fee, \$690.00 (\$345.00):	\$690.00
Number of Claims in excess of 20 at \$18.00 (\$9.00) each:	\$
Number of Independent Claims in excess of 3 at \$78.00 (\$39.00) each:	\$.00
Multiple Dependent Claim Fee at \$260.00 (\$130.00):	\$
Total Filing Fee:	\$.00

☐ Assignment Recording Fee \$40.00:

☒ TOTAL: \$690.00

☐ This application is being filed within the ____ month following the expiration of the term originally set therefor in the prior application. This is a petition to request a -month extension of time. A check covering the cost of the petition is enclosed.

☒ Oath or Declaration and Power of Attorney

☐ New ☐ signed ☐ unsigned

☒ Copy from a prior application (37 C.F.R. 1.63(d)) (See also Preliminary Amendment)

Deletion of Inventors

☐ Signed Statement attached deleting inventor(s) named in the prior application (37 C.F.R. 1.63(d)(2) and 1.33(b))

Power of Attorney or Correspondence Address Change

X Power of attorney and/or correspondence address was changed during prosecution of the prior application. The power of attorney is to **THOMAS J. KOWALSKI, REG. NO. 32,147**. The new correspondence address is indicated above.

— Signed Statement attached deleting inventor(s) named in the prior application (37 C.F.R. 1.63(d)(2) and 1.33(b))

X Incorporation by Reference (for continuation or divisional application)

The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

X A Preliminary Amendment is as follows.

Page 1, underneath the Title, please insert:

--This application is a divisional of U.S. application Serial No. 09/713,053, filed December 16, 1998, which is a continuation of PCT/FR97/01115, filed June 23, 1997.--

Prior to examination and fee calculation, please amend the application as follows:

— New formal drawings are enclosed.

X Certified copy of each foreign priority application on which the claim for priority under 35 U.S.C. 119 is based was filed in prior U.S. applications serial nos.

PATENT
454313-2200.1

Application No.
0608242

Filed
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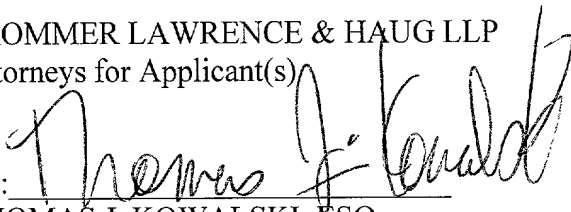
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Please charge any additional fees required for the filing of this application or credit any overpayment to Deposit Account No. 50-0320.

Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicant(s)

By:


THOMAS J. KOWALSKI, ESQ.

Reg. No. 32,147

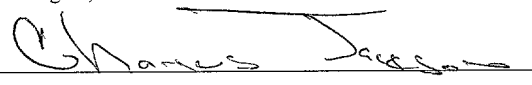
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IN THE UNITED STATES PATENT AND TRADEMARK

APPLICATION FOR LETTERS PATENT

TITLE: CANINE HERPESVIRUS BASED RECOMBINANT LIVE
VACCINE, IN PARTICULAR AGAINST CANINE DISTEMPER,
RABIES OR THE PARAINFLUENZA 2 VIRUS

INVENTORS: Jean-Christophe AUDONNET and Philippe BAUDU

CLAIMS: 16 (2 independent)

William S. Frommer
Registration No. 25,506
FROMMER, LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, New York 10151
Tel: (212) 588-0800

Recombinant live vaccine based on canine herpesvirus, in particular against Carré's disease, rabies or the parainfluenza virus type 2.

5 The present invention relates to vaccines, preferably for dogs, produced from recombinant canine herpesviruses, and to the methods for obtaining and preparing these recombinant viruses. The present invention relates more especially to recombinant canine
10 herpesviruses comprising an expression cassette for one or more foreign gene(s).

Canine herpesvirosis is caused by the canine herpesvirus (CHV). The canine herpesvirus (CHV) is classified in the Alphaherpesvirinae family. This
15 herpesvirus is a major pathogen for neonatal puppies. Canine herpesvirosis manifests itself chiefly in a haemorrhagic disease in puppies, and in a benign disease of the upper respiratory apparatus in adult dogs. There are at present no vaccines for protecting
20 puppies against canine herpesvirosis.

Moreover, domestic dogs are exposed to numerous other diseases, and the development of a vaccinal vector capable of expressing different antigens of canine pathogenic agents would enable the efficacy of
25 vaccination programmes to be simplified and improved, especially for puppies in breeding kennels. Among pathogenic agents of importance for dogs, the Carré's disease virus, the Rubarth's hepatitis virus, the rabies virus, the canine parvoviro-sis virus, the canine coronavirus, the parainfluenza virus type 2, *Bordetella*
30 *bronchiseptica*, *Borrelia burgdorferi*, *Leptospira* spp. and *Leishmania infantum* may be mentioned.

Little is known about the CHV virus genome. The genomic organization of this virus was published only
35 recently (Rémond M. et al. J. Gen. Virol. 1996. 77. 37-48), and the genes for the three major glycoproteins gB, gC and gD, as well as a gene designated CHV ORF2, have been described (K. Limbach et al. J. Gen. Virol. 1994. 75. 2029-2039).

Following their work on CHV, the inventors have succeeded in determining several regions which are non-essential for replication *in vitro*, which have proved useful for the construction of recombinant CHV viruses.

5 The inventors are hence in a position to put forward for the first time the CHV virus as a vaccination vector for dogs. It was found that the vaccinal vectors according to the invention had particular advantages for the vaccination of dogs. In effect, the canine
10 herpesvirus is very species-specific and possesses a large genome containing several potential insertion sites and permitting the simultaneous insertion of several expression cassettes for foreign genes. This affords the possibility of vaccinating dogs at the same
15 time against different canine pathogenic agents using a single recombinant virus.

The main objective of the invention is to provide a vaccinal vector permitting the expression of immunogens of canine pathogens for the purpose of
20 protecting dogs against the main canine infectious diseases.

Another objective of the invention is to provide such a vector permitting the vaccination of dogs, and especially puppies having maternal
25 antibodies, via the mucosal, in particular the oral, nasal or conjunctival, route.

Yet another objective of the invention is to provide such a vector which permits vaccination at the same time against herpesvirosis in puppies.

30 Hence the subject of the present invention is a recombinant live vaccine using as vector a canine herpesvirus comprising and expressing at least one nucleotide sequence coding for a polypeptide, this sequence being inserted into a site which is non-
35 essential for replication *in vitro*.

The inventors have isolated and analysed a genomic fragment of the CHV virus, on which they have characterized 5 open reading frames (ORF1 to ORF5), among which two (ORF3 and ORF5) have proved to be non-

essential for replication *in vitro*. Moreover, the inventors have found that other regions of the CHV genome could also be used to insert foreign genes. These insertion sites are: thymidine kinase gene (CHV TK ORF) (Rémond M. et al. Virus Research. 1995. 39. 341-354.) and sequence situated between the CHV ORF19 and the CHV ORF22 (Rémond M. et al. J. Gen. Virol. 1996. 76. 37-48). These sites are described more precisely in the examples of the present invention.

Preferably, the inserted sequence codes for an antigenic polypeptide, and preferentially for an antigenic polypeptide of a canine pathogenic agent. It is also possible to insert the sequences coding for immunomodulatory proteins such as cytokines. According to an advantageous variant, it is possible to use in combination a sequence coding for a cytokine, or the like, and a sequence coding for an antigen. If need be, several cytokine sequences can be used in combination with one another, optionally in combination with one or more sequences coding for antigens.

The insertion into the sites is carried out by simple insertion (without deletion), or after partial or total deletion of the ORF or ORFs used as insertion sites.

As a parent virus for the construction of recombinant CHV viruses, it is possible to use, in particular, the CHV strain F205 which was isolated by L. Carmichael (Proc. Soc. Exp. Biol. Med. 1965. 120. 644-650).

For the expression of foreign genes inserted into the CHV genome according to the present invention, it will be preferable to use a strong eukaryotic promoter such as, preferentially, a cytomegalovirus (CMV) immediate-early (IE) promoter. CMV IE promoter is understood to mean, in particular, a fragment such as is given in the examples, as well as the subfragments thereof retaining the same promoter activity. The CMV IE promoter can be the human (HCMV IE) promoter or the murine (MCMV IE) promoter, or alternatively a CMV IE

promoter of another origin, for example rat, guinea pig or porcine CMV.

At least two nucleotide sequences may be inserted into one site under the control of different promoters. The latter may be, in particular, CMV IE promoters of different origins.

According to an advantageous development of the invention, another promoter is used in combination with the CMV IE promoter in such a way that the two promoters have their 5' ends adjacent and that the transcriptions initiated from these two promoters take place in opposite directions. This particular arrangement enables two nucleotide sequences to be inserted into the same site, one under the control of the CMV IE promoter and the other under that of the promoter used in combination with it. This construction is noteworthy from the fact that the presence of the CMV IE promoter, and in particular of its enhancer portion, can activate the transcription induced by the promoter used in combination. As a promoter used in combination, there may be mentioned, for example, a CMV promoter of different species from the first promoter. It is also possible to envisage other promoters, such as the Marek's disease virus (MDV) RNA1.8 promoter (G. Bradley et al. J. Virol. 1989. 63. 2534-2542).

The nucleotide sequence inserted into the CHV vector in order to be expressed can be any sequence coding for an antigenic polypeptide of a canine pathogenic agent capable, when expressed under the favourable conditions obtained by the invention, of bringing about an immunization leading to an effective protection of the vaccinated animal against the pathogenic agent. The nucleotide sequences coding for the antigens of interest for a given disease, in particular the viral, bacterial or parasitic diseases mentioned above, may hence be inserted under the conditions described by the present invention.

The typical case of the invention is the insertion of at least one nucleotide sequence coding

appropriately for a polypeptide of the Carré's disease virus (canine distemper virus = CDV), and preferably for the CDV polypeptide HA (Sidhu M. et al., Virology. 1993. 193. 66-72) or for the CDV polypeptide F (Barrett T. et al. Virus Research. 1987. 8. 373-386). It is also possible to insert both of these genes together into the CHV vector. A recombinant live vaccine bringing about protection against Carré's disease is thereby obtained.

Other preferred cases of the invention are the insertion of nucleotide sequences coding for antigens or fragments of antigens of the rabies virus, especially the G gene (Patents FR-A-2,515,685 and EP-A-162,757), of the canine parvovirus (VP2 gene) (Parrish C. et al. J. Virol. 1991. 65. 6544-6552) or of the parainfluenza virus type 2 (HA and/or F genes). It is also possible to insert sequences coding for *Borrelia burgdorferi* antigens, especially the genes coding for the OspA and OspB antigens (Bergström S. et al. Mol. Microbiol. 1989. 3. 479-486).

A typical case of the invention is a vaccine comprising a nucleotide sequence coding for an antigen of the Carré's disease virus under the control of CMV IE, and a nucleotide sequence coding for an antigen of another canine viral disease, in particular the ones mentioned above, under the control of the other promoter.

Naturally, the heterologous sequences and their associated promoters may be inserted more conventionally in tandem into the insertion locus, that is to say according to the same transcription direction.

The expression of several heterologous genes inserted into the insertion locus can also be possible by insertion of a sequence known as an "IRES" (internal ribosome entry site) originating, in particular, from a picornavirus such as the swine vesicular disease virus (SVDV; B.-F. Chen et al., J. Virology, 1993, 67, 2142-2148), the encephalomyocarditis virus (EMCV; R.J.

Kaufman et al., *Nucleic Acids Research*, 1991, 19, 4485-4490) or the aphthous fever virus (FMDV; N. Luz and E. Beck, *J. Virology*, 1991, 65, 6486-6494), or alternatively of another origin. The content of these
5 three papers is incorporated by reference. The cassette for expression of two genes would hence have the following minimum structure: promoter - gene 1 - IRES - gene 2 - polyadenylation signal. The recombinant live vaccine according to the invention may hence comprise,
10 inserted into the insertion locus, an expression cassette comprising in succession a promoter, two or more genes separated in pairs by an IRES, and a polyadenylation signal.

In addition to the insertion into the locus
15 according to the invention, it is possible to carry out one or more other insertions, one or more mutations or one or more deletions elsewhere in the genome. In all cases, insertion into a locus other than the one described in the invention enables other genes to be
20 expressed.

The use of the recombinant viruses according to the invention enables dogs to be protected against one or more of the diseases mentioned above, and at the same time against canine herpesviro-sis.

25 The subject of the present invention is also a polyvalent vaccine formula comprising, as a mixture or to be mixed, at least two recombinant live vaccines as defined above, these vaccines comprising different inserted sequences isolated, in particular, from
30 different pathogens. These vaccine formulae contain dosages and/or vehicles which are suited to the administration route.

The subject of the present invention is also CHV viruses modified in at least one of the sites
35 indicated.

Its subject is also a method of vaccination, especially of dogs, in which an effective amount of a vaccine as defined above is administered via any parenteral or mucosal route, but preferably via the

mucosal, in particular the oral and/or nasal, route. The vaccinal dose will preferably be between 10^2 CCID50 and 10^7 CCID50. Preferably, the dose for the parenteral route will be between 10^4 CCID50 and 10^7 CCID50, and for
5 the oral and/or nasal route, between 10^2 CCID50 and 10^5 CCID50. As defined, the vaccine is effective in general after a single administration via the oral and/or nasal route. However, repeated administrations may be necessary.

10 The subject of the present invention is also the DNA fragments comprising all or part of the sequence defined by positions 1 to 6216 on SEQ ID No. 1 (Figure No. 1), in particular all or part of the ORF3
15 site defined and/or of the flanking sequences located upstream and downstream of this site, which fragments will be useful as flanking arms for the techniques of homologous recombination with the genome of the CHV virus chosen as parent virus. Naturally, the invention also relates to the variants of these fragments which
20 correspond to the equivalent sequences of the other strains of CHV. The expert is entirely free to choose the regions serving as flanking arms in connection with the type of insertion (with or without deletion) or of deletion (partial or total) chosen. Generally speaking,
25 the flanking arms may thus have from 100 to 800 base pairs, but can be larger if necessary.

A further subject of the invention is a method of preparation of the vectors and vaccines according to the invention, as emerges from the description of the
30 vaccines, by insertion of genes of interest into the insertion site.

The invention will now be described in greater detail by means of non-limiting examples of implementation, taken with reference to the drawing,
35 wherein:

Figure 1: Sequence of the CHV region (6216 base pairs) and translation of the different open reading frames (ORFs) present in this sequence (ORF1 to ORF5).

- Figure 2: Plasmid pPB200 (donor plasmid for the insertion of expression cassettes into the CHV ORF3 site).
- 5 Figure 3: Construction of the plasmid pPB202 (donor plasmid for the insertion of expression cassettes into the CHV ORF5 site).
- 10 Figure 4: Construction of the plasmid pPB204 (donor plasmid for the insertion of expression cassettes into the CHV TK site).
- 15 Figure 5: Construction of the plasmid pPB206 (donor plasmid for the insertion of expression cassettes into the site situated between the CHV ORF19 and CHV ORF22 genes).
- 20 Figure 6: Construction of the plasmid pPB208 (expression cassette for the CDV HA gene).
- Figure 7: Construction of the plasmid pPB210 (expression cassette for the CDV F gene).
- 25 Figure 8: Construction of the plasmid pPB213 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF3 site).
- 30 Figure 9: Construction of the plasmid pPB214 (donor plasmid for the insertion of the cassette for the expression of the CDV F gene into the CHV ORF3 site).
- Figure 10: Plasmid pPB200'
- Figure 11: Construction of the plasmid pPB212 (cassette for the expression of the rabies virus G gene).
- 35 Figure 12: Construction of the plasmid pPB125 (donor plasmid for the insertion of the cassette for the expression of the rabies virus G gene into the CHV ORF3 site).

Figure 13: Construction of the plasmid pPB216 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF5 site).

5 **Figure 14:** Construction of the plasmid pPB217 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV TK site).

10 **Figure 15:** Construction of the plasmid pPB218 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the site situated between the CHV ORF19 and CHV ORF22 genes).

15 SEQ ID sequence listing for the constructions in the insertion sites of the CHV vector:

SEQ ID No. 1 Complete sequence of the CHV ORF1 →
ORF5 region depicted in Figure 1

20 SEQ ID No. 2 ORF1 amino acid sequence of Figure 1
SEQ ID No. 3 ORF2 amino acid sequence of Figure 1
SEQ ID No. 4 ORF3 amino acid sequence of Figure 1
SEQ ID No. 5 ORF4 amino acid sequence of Figure 1
SEQ ID No. 6 (Partial) ORF5 amino acid sequence of
25 Figure 1

SEQ ID No. 7 Oligonucleotide JCA070
SEQ ID No. 8 Oligonucleotide JCA071
SEQ ID No. 9 Oligonucleotide JCA072
SEQ ID No. 10 Oligonucleotide JCA073

30 SEQ ID No. 11 Oligonucleotide JCA074
SEQ ID No. 12 Oligonucleotide JCA075
SEQ ID No. 13 Oligonucleotide JCA076
SEQ ID No. 14 Oligonucleotide JCA077
SEQ ID No. 15 Oligonucleotide JCA078

35 SEQ ID No. 16 Oligonucleotide JCA079
SEQ ID No. 17 Oligonucleotide JCA080
SEQ ID No. 18 Oligonucleotide JCA081
SEQ ID No. 19 Oligonucleotide JCA082
SEQ ID No. 20 Oligonucleotide JCA083

SEQ ID No. 21 Oligonucleotide JCA084
SEQ ID No. 22 Oligonucleotide JCA085
SEQ ID No. 23 Oligonucleotide PB088
SEQ ID No. 24 Oligonucleotide PB089
5 SEQ ID No. 25 Oligonucleotide JCA086
SEQ ID No. 26 Oligonucleotide JCA087
SEQ ID No. 27 Oligonucleotide JCA088
SEQ ID No. 28 Oligonucleotide JCA089
SEQ ID No. 29 Oligonucleotide JCA090
10 SEQ ID No. 30 Oligonucleotide JCA091

EXAMPLES

All the constructions of plasmids were carried out using the standard techniques of molecular biology described by Sambrook J. et al. (*Molecular Cloning: A Laboratory Manual*. 2nd Edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York. 1989). All the restriction fragments used for the present invention were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

The virus used as parent virus is the canine herpesvirus strain F205 (also known as the Carmichael strain). This strain was obtained from Dr. L. Carmichael (Cornell University, NY), who isolated it and described its biological characteristics (*Proc. Soc. Exp. Biol. Med.* 1965. 120. 644-650). The conditions of culture of this virus are as follows: MDCK (Madin-Darby canine kidney ATCC CCL34) cells cultured in Eagle's minimum essential medium (MEM medium) are inoculated with the CHV strain F205 using a multiplicity of infection of 1. The infected cells are then incubated at 37°C for approximately 36 hours until a complete cytopathic effect is seen.

35 Example 1: Extraction of canine herpesvirus DNA

After culture, the supernatant and the lysed cells are harvested, and the whole of the viral suspension is centrifuged at 1000 g for 10 minutes at +4°C to remove cell debris. The viral particles are

then harvested by ultracentrifugation at 400,000 g for 1 hour at +4°C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral suspension is treated with
5 proteinase K (100 µg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a phenol/chloroform mixture and thereafter precipitated with 2 volumes of absolute ethanol. After one night at
10 -20°C, the precipitated DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water.

15 **Example 2: Cloning and characterization of the CHV ORF1 - ORF5 region**

The purified genomic DNA of the CHV virus strain F205 was digested with the restriction enzymes ScaI and XhoI, and the approximately 6200-bp ScaI-XhoI
20 fragment was cloned into the vector pBlueScript SKII+ (Stratagene Ref. 212205), previously digested with ScaI and XhoI, to give the plasmid pPB154.

The XhoI-ScaI fragment cloned into plasmid pPB154 was sequenced completely on both strands to
25 generate the 6216-bp sequence of Figure 1 (SEQ ID No. 1).

Several open reading frames larger than 65 amino acids in size were identified on this sequence (Figure 1):

30 The first reading frame (ORF1) (positions 1353 - 157) occurs on the complementary strand and codes for a polypeptide of 398 amino acids (SEQ ID No. 2).

The second reading frame (ORF2) (positions 1708 - 2970) codes for a polypeptide of 420 amino acids
35 (SEQ ID No. 3).

The third reading frame (ORF3) (positions 3040 - 4242) codes for a polypeptide of 400 amino acids (SEQ ID No. 4).

The fourth reading frame (ORF4) (positions 4374 - 5753) codes for a polypeptide of 459 amino acids (SEQ ID No. 5).

The fifth reading frame (ORF5) (positions 5872 - 6216) is incomplete and codes for a truncated protein of 115 amino acids (SEQ ID No. 6).

The different open reading frames are collated in the table below:

Open reading frame	Beginning - End (positions in Figure 1)	Size in amino acids
ORF 1	1353 - 157	398 aa
ORF 2	1708 - 2970	420 aa
ORF 3	3040 - 4242	400 aa
ORF 4	4374 - 5753	459 aa
ORF 5	5872 - 6216	115 aa

Example 3: Construction of plasmid pPB200 for the insertion of expression cassettes into the CHV ORF3 site (Figure 2)

Plasmid pPB154 (9121 bp) (Example 2) was digested with *Hind*III and *Spe*I to isolate the 620-bp *Hind*III-*Spe*I fragment (fragment A). Plasmid pPB154 was digested with *Eco*RI and *Spe*I to isolate the 659-bp *Eco*RI-*Spe*I fragment (fragment B). The fragments A and B were ligated together with the vector pGEM4Z (Promega Ref. P2161), previously digested with *Eco*RI and *Hind*III, to give the plasmid pPB199 (4096 bp). Plasmid pPB199 was then digested with *Spe*I, treated with alkaline phosphatase and then ligated with the multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA070 (33 mer) (SEQ ID No. 7)

5' CTAGTCCAGCAAGGTGGATCGATATCGGGCCCA 3'

JCA071 (33 mer) (SEQ ID No. 8)

5' CTAGTGGGCCCCGATATCGATCCACCTTGCTGGA 3'

to give plasmid pPB200 (4129 bp).

Example 4: Construction of plasmid pPB202 for the insertion of expression cassettes into the CHV ORF5 site (Figure 3)

The sequence of the CHV ORF5 gene was published recently (Limbach K. et al. J. Gen. Virol. 1994. 75. 2029-2039). A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:

JCA072 (22 mer) (SEQ ID No. 9)

10 5'CAGCTTTATGTTTTTATTGTTC 3'

JCA073 (29 mer) (SEQ ID No. 10)

5'AAAGAATTCTACAACGTGTTTAATAAAGAC 3'

to obtain a 751-bp PCR fragment containing the complete CHV ORF2 gene. This fragment was digested with BglII and EcoRI to isolate a 709-bp BglII-EcoRI fragment. This fragment was ligated with the vector pGEM4Z (Promega Ref. P2161), previously digested with EcoRI and BamHI, to give the plasmid pPB201 (3559 bp). Plasmid pPB201 was then digested with ScaI and PvuII and thereafter ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA074 (36 mer) (SEQ ID No. 11)

5'ACTCCAGCTACATGGGATATCGGGCCCATCGATCAG 3'

25 JCA075 (36 mer) (SEQ ID No. 12)

5'CTGATCGATGGGCCCCGATATCCCATGTAGCTGGAGT 3'

to give plasmid pPB202 (3395 bp).

Example 5: Construction of plasmid pPB204 for the insertion of expression cassettes into the CHV TK site (Figure 4)

The sequence of the CHV thymidine kinase (TK) gene was published recently (Rémond M. et al. Virus Research. 1995. 39. 341-354). A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:

JCA076 (35 mer) (SEQ ID No. 13)

5'AGCGTTAACCTCAAAAGCCAAATTTACACTTCCCG 3'

JCA077 (38 mer) (SEQ ID No. 14)

5'CCCAAGCTTTTCTAAAGCCCATTATAAATAATAAATG 3'

to obtain a 1030-bp PCR fragment containing the thymidine kinase (TK) gene. This fragment was digested with *HpaI* and *HindIII* to isolate a 1019-bp *HpaI*-*HindIII* fragment. This fragment was ligated with the vector pSP73 (Promega Ref. P2221), previously digested with *EcoRV* and *HindIII*, to give the plasmid pPB203 (3423 bp).

Plasmid pPB203 was then digested with *EcoRI* and *StyI* and thereafter ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA078 (36 mer) (SEQ ID No. 15)

5'AATTCCTCAGCTACATGGGATATCGGGCCCATCGATC 3'

JCA079 (36 mer) (SEQ ID No. 16)

5'CAAGGATCGATGGGCCCCGATATCCCATGTAGCTGGG 3'

to give plasmid pPB204 (3399 bp).

Example 6: Construction of plasmid pPB206 for the insertion of expression cassettes into the site situated between the CHV ORF19 and ORF22 genes (Figure 5)

The sequence of the intergenic region corresponding to the natural deletion of the genes coding for the large subunit ("RR1" gene) and for the small subunit ("RR2" gene) of ribonucleotide reductase was published recently (Rémond M. et al. J. Gen. Virol. 1996, 77. 37-48). According to the nomenclature used by Rémond et al., the deletion of these two genes occurs between the open reading frames designated CHV "orf19" and CHV "orf22" [designated herein ORF19 and ORF 22, respectively]. A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:

JCA080 (36 mer) (SEQ ID No. 17)

5'GGAGATCTAGTAAATTAAATAGTAATTCATTTAATG 3'

JCA081 (33 mer) (SEQ ID No. 18)

5'CAGTCGCGAAGATGAAAATAAAATCCATCGAAG 3'

to obtain a 720-bp PCR fragment containing the intergenic region corresponding to the natural deletion of the CHV ORF19 and ORF22 genes. This fragment was digested with *SpeI* and *NruI* to isolate a 709-bp *SpeI*-*NruI* fragment. This fragment was ligated with the vector pGEM4Z (Promega Ref. P2161), previously digested with *SmaI* and *XbaI*, to give the plasmid pPB205 (3572 bp). Plasmid pPB205 was then digested with *MfeI* and thereafter partially digested with *SspI* in order to isolate the 3512-bp *MfeI*-*SspI* fragment. This fragment was then ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA082 (38 mer) (SEQ ID No. 19)
5'AATTGGCAGCTACATGGGATATCGGGCCCATCGATAAT 3'

JCA083 (34 mer) (SEQ ID No. 20)
5'ATTATCGATGGGCCCCGATATCGGATGTAGCTGGC 3'

to give plasmid pPB206 (3548 bp).

Example 7: Isolation of the genomic RNA of the CDV strain Onderstepoort and cloning of the complementary DNA coding for the HA and F genes

The CDV strain Onderstepoort (Mitchell W. et al. J. Virol. Meth. 1987. 18. 121-131) was cultured on MDCK (Madin-Darby canine kidney) cells in DMEM medium (Gibco). After purification of the virus, the genomic viral RNA was isolated using the guanidinium thiocyanate/phenol-chloroform extraction technique (Chomczynski P. and Sacchi N., Anal. Biochem. 1987. 162. 156-159). Specific oligonucleotides (containing restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized in such a way as to cover completely the coding regions of the genes which were to be amplified (HA and F genes, respectively). The reverse transcription (RT) reaction and polymerase chain amplification (PCR) were performed according to the standard techniques (Sambrook J. et al. 1989). Each RT-PCR reaction was carried out with a pair of specific amplimers and taking as template the extracted viral genomic RNA. The amplified comple-

mentary DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with the restriction enzymes and cloned into the appropriate vector.

5

7.1. Construction of the CDV HA expression cassette (pPB208) (Figure 6)

The plasmid pCMVB (Clontech Ref. 6177-1) was digested with *EcoRI* and *NotI* to isolate an 818-bp
10 *EcoRI-NotI* fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

An RT-PCR reaction was carried out with the genomic RNA of the CDV virus (strain Onderstepoort) and
15 with the following oligonucleotides:

JCA084 (32 mer) (SEQ ID No. 21)

5'TTGCGGCCGCGCATGCTCCCCTACCAAGACAAGG 3'

JCA085 (28 mer) (SEQ ID No. 22)

5'TTGGTACCTTAACGGTTACATGAGAATC 3'

20 to obtain a 1837-bp PCR fragment containing the CDV HA gene. This fragment was digested with *NotI* and *KpnI* to isolate a 1826-bp *NotI-KpnI* fragment (fragment B).

The fragments A and B were ligated together with the vector pGEM-7Zf+ (Promega Cat # P2251),
25 previously digested with *EcoRI* and *KpnI*, to give the plasmid pPB207 (5634 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:

PB088 (30 mer) (SEQ ID No. 23)

30 5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'

PB089 (32 mer) (SEQ ID No. 24)

5'TTGGGTACCGGATCCGAAAAACCTCCCACAC 3'

to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene.
35 This fragment was digested with *KpnI* to isolate a 233-bp *KpnI-KpnI* fragment. This fragment was then ligated with plasmid pPB207, previously digested with *KpnI*, to give plasmid pPB208 (5867 bp).

7.2. Construction of the CDV F expression cassette (pPB210) (Figure 7)

Plasmid pCMVB (Clontech Ref. 6177-1) was digested with *EcoRI* and *NotI* to isolate an 818-bp *EcoRI-NotI* fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

An RT-PCR reaction was carried out with the genomic RNA of the CDV virus (strain Onderstepoort) and with the following oligonucleotides:

JCA086 (34 mer) (SEQ ID No. 25)

5'TTGCGGCCGCATGCACAGGGGAATCCCCAAAAGC 3'

JCA087 (28 mer) (SEQ ID No. 26)

5'TTGGTACCTCAGAGTGATCTCACATAGG 3'

to obtain a 2011-bp PCR fragment containing the CDV F gene. This fragment was digested with *NotI* and *KpnI* to isolate a 2000-bp *NotI-KpnI* fragment (fragment B). The fragments A and B were ligated together with the vector pGEM-7Zf+ (Promega Ref. P2251), previously digested with *EcoRI* and *KpnI*, to give the plasmid pPB209 (5808 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:

PB088 (30 mer) (SEQ ID No. 23)

25 5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'

PB089 (32 mer) (SEQ ID No. 24)

5'TTGGGTACCGGATCCGAAAAACCTCCCACAC 3'

to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene. This fragment was digested with *KpnI* to isolate a 233-bp *KpnI-KpnI* fragment. This fragment was then ligated with plasmid pPB209, previously digested with *KpnI*, to give plasmid pPB210 (6041 bp).

35 **Example 8: Construction of the donor plasmid pPB213 for the insertion of the CDV HA expression cassette into the CHV ORF3 site (Figure 8)**

Plasmid pPB208 (Example 7.1.) was digested with *ApaI* and *ClaI* to isolate a 2920-bp *ApaI-ClaI* fragment

containing the cassette for the expression of the CDV virus HA gene. This fragment was then ligated with plasmid pPB200 (Example 3), previously digested with ApaI and ClaI, to give plasmid pPB213 (7043 bp). This
5 plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF3 site.

**Example 9: Construction of the donor plasmid pPB214 for the insertion of the CDV F expression cassette into the
10 CHV ORF3 site (Figure 9)**

Plasmid pPB210 (Example 7.2.) was digested with ApaI and ClaI to isolate a 3100-bp ApaI-ClaI fragment containing the cassette for the expression of the CDV virus F gene. This fragment was then ligated with
15 plasmid pPB200 (Example 3), previously digested with ApaI and ClaI, to give plasmid pPB214 (7217 bp). This plasmid permits the insertion of the cassette for the expression of the CDV F gene into the CHV ORF3 site.

20 Example 10: Construction of the donor plasmid pPB215 for the insertion of the cassette for the expression of the rabies virus G gene into the CHV ORF3 site (Figures 10, 11 and 12)

Plasmid pPB199 (Example 3) was digested with
25 SpeI, treated with alkaline phosphatase and then ligated with the multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA088 (39 mer) (SEQ ID No. 27)

5'CTAGTCCAGCAAGGTGTCGACGGATCGATATCGGGCCCA 3'

30 JCA089 (39 mer) (SEQ ID No. 28)

5'CTAGTGGGCCCCGATATCGATCCGTCGACACCTTGCTGGA 3'

to give plasmid pPB200' (4135 bp) (Figure 10).

Plasmid pCMVB (Clontech Ref. 6177-1) was digested with EcoRI and NotI to isolate an 818-bp
35 EcoRI-NotI fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

According to the technical procedures already described for the CDV virus (Example 7), the RNA of the

rabies virus ERA strain was extracted and purified from a culture of rabies virus-infected Vero cells. An RT-PCR reaction was then carried out (see Example 7) with the genomic RNA of the rabies virus (strain ERA) and
5 with the following oligonucleotides:

JCA090 (31 mer) (SEQ ID No. 29)

5'TTGCGGCCGCATGGTTCCTCAGGCTCTCCTG 3'

JCA091 (31 mer) (SEQ ID No. 30)

5'TTGGTACCTCACAGTCTGGTCTCACCCCCAC 3'

10 to obtain a 1597-bp PCR fragment containing the rabies virus G gene (Patents FR-A-2,515,685 and EP-A-162,757). This fragment was digested with *NotI* and *KpnI* to isolate a 1586-bp *NotI*-*KpnI* fragment (fragment B). The fragments A and B were ligated together with the vector
15 pSP73 (Promega Ref. P2221), previously digested with *EcoRI* and *KpnI*, to give the plasmid pPB211 (4852 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:

PB088 (30 mer) (SEQ ID No. 23)

20 5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'

PB089 (32 mer) (SEQ ID No. 24)

5'TTGGGTACCGGATCCGAAAAACCTCCCACAC 3'

to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene.
25 This fragment was digested with *KpnI* to isolate a 233-bp *KpnI*-*KpnI* fragment. This fragment was then ligated with plasmid pPB211, previously digested with *KpnI*, to give plasmid pPB212 (5085 bp) (Figure 11). Plasmid pPB212 was digested with *EcoRV* and *SalI* to isolate a
30 2664-bp *EcoRV*-*SalI* fragment containing the cassette for the expression of the rabies virus G gene. This fragment was then ligated with plasmid pPB200' (see above), previously digested with *EcoRV* and *SalI*, to give plasmid pPB215 (6790 bp) (Figure 12).

35

Example 11: Construction of the donor plasmid pPB216 for the insertion of the CDV HA expression cassette into the CHV ORF5 site (Figure 13)

Plasmid pPB208 (Example 7.1.) was digested with *Sma*I and *Apa*I to isolate a 2909-bp *Sma*I-*Apa*I fragment containing the cassette for the expression of the CDV HA gene. This fragment was then ligated with plasmid pPB202 (Example 4), previously digested with *Eco*RV and *Apa*I, to give plasmid pPB216 (6291 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF5 site.

10 **Example 12: Construction of the donor plasmid pPB217 for the insertion of the CDV HA expression cassette into the CHV TK site (Figure 14)**

Plasmid pPB208 (Example 7.1.) was digested with *Apa*I and *Cla*I to isolate a 2920-bp fragment containing the cassette for the expression of the CDV HA gene. This fragment was ligated with plasmid pPB204 (Example 5), previously digested with *Apa*I and *Cla*I, to give plasmid pPB217 (6316 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV TK site.

Example 13: Construction of the donor plasmid pPB218 for the insertion of the CDV HA expression cassette into the site situated between the CHV ORF19 and CHV ORF22 genes (Figure 15)

Plasmid pPB208 (Example 7.1.) was digested with *Apa*I and *Cla*I to isolate a 2920-bp fragment containing the cassette for the expression of the CDV HA gene. This fragment was ligated with plasmid pPB206 (Example 6), previously digested with *Apa*I and *Cla*I, to give plasmid pPB218 (6462 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the site situated between the CHV ORF19 and CHV ORF22 genes.

Example 14: Isolation of the recombinant virus vCHV01 containing the cassette for the expression of the CDV HA gene in the CHV ORF3 site.

Plasmid pPB213 (see Example 8) was linearized
5 by digestion with *HindIII*, extracted with a phenol-chloroform mixture, precipitated with absolute ethanol and then taken up in sterile water.

MDCK cells forming a well-established cell lawn in a Petri dish (Corning 4.5 cm in diameter) were then
10 transfected with the following mixture:
1 μ g of linearized plasmid pPB213 + 5 μ g of CHV viral DNA in 300 μ l of MEM medium and 100 μ g of LipofectAMINE (Gibco-BRL Cat# 18324-012) diluted in 300 μ l of medium (final volume of the mixture = 600 μ l). These 600 μ l
15 were then diluted in 3 ml (final volume) of MEM medium and spread over 3×10^6 MDCK cells. The mixture was left in contact with the cells for 5 hours, then removed and replaced by 5 ml of culture medium. The cells were then left in culture for 24 hours at +37°C. After 24 hours
20 to 48 hours of culture, 1 ml of culture supernatant was harvested, and several dilutions of this supernatant were used to infect other MDCK cells (cultured in Petri dishes (Corning 4.5 cm in diameter)) so as to obtain isolated plaques, each dish being infected with 1 ml of
25 a dilution of the initial supernatant. After contact for 1 hour at 37°C, the infection medium was removed and replaced by 5 ml of MEM medium containing 1% of agarose, kept supercooled at 42°C. When the agarose had solidified, the dishes were incubated for 48 hours at
30 37°C in a CO₂ incubator until plaques were seen. The agarose layer was then removed, and a transfer of the viral plaques was carried out onto a sterile nitrocellulose membrane of the same diameter as the Petri dish used for culturing. This membrane was itself
35 transferred onto another nitrocellulose membrane so as to obtain an inverted "copy" of the first transfer. The plaques transferred onto this second copy were then hybridized, according to the standard techniques known to a person skilled in the art, with a 1842-bp NotI-

NotI fragment of the CDV HA gene, obtained by digestion of plasmid pPB208 (Example 7.1.), labelled with digoxigenin (DNA Labelling kit, Boehringer Mannheim, CAT # 1175033). After hybridization, washes and
5 contacting with the visualization substrate, the nitrocellulose membrane was placed in contact with an autoradiographic film. The images of positive hybridization on this membrane indicated which plaques were the ones which contained recombinant CHV viruses
10 which had inserted the CDV HA cassette. The plaques corresponding to these positive plaques were cut out under sterile conditions from the first nitrocellulose membrane, placed in an Eppendorf tube containing 0.5 ml of MEM medium and sonicated to release the virions from
15 the membrane. The medium contained in the Eppendorf tube was then diluted in MEM medium, and the dilutions thereby obtained were used to infect further cultures of MDCK cells. A 100% pure recombinant virus containing the HCMV-IE/CDV HA/polyA cassette inserted into the
20 ORF3 site was thereby isolated after 3 cycles of purification, and was called vCHV01. The homology of the recombination was verified by PCR using oligonucleotides situated on each side of the insertion site. The absence of reorganization on the genome of
25 the recombinant virus vCHV01, other than in the recombination region, was verified by the Southern blot technique.

Example 15: Isolation of recombinant CHV viruses expressing various foreign genes

30 According to the procedure described in Example 14, the construction of different recombinant CHV viruses is carried out using the donor plasmids described in Examples 9 to 13.

35

Example 16: Preparation of the vaccines

To prepare a vaccine, the recombinant viruses obtained in Examples 14 and 15 are cultured on MDCK cells. Harvesting of the recombinant virus takes place

when the cytopathic effect is complete. The lysed cells and the culture supernatant are harvested. After clarification of the cell lysate to remove cell debris, the viral solution is titrated. The viral solution is
5 then diluted in a stabilizing solution for lyophilization, distributed on the basis of one vaccinal dose (10^2 CCID₅₀ to 10^7 CCID₅₀) per vial and lastly lyophilized. The viral solution can then be frozen if necessary.

10

Example 17: Vaccination methods

According to the preferred mode of vaccination, the vaccine obtained according to the invention is redissolved or thawed, and then administered via the
15 parenteral or mucosal route, but preferably via the mucosal, in particular the oral and/or nasal, route. The vaccinal dose will preferably be between 10^2 CCID₅₀ and 10^7 CCID₅₀. Preferably, the dose for the parenteral route will be between 10^4 CCID₅₀ and 10^7 CCID₅₀, and for
20 the oral and/or nasal route, between 10^2 CCID₅₀ and 10^5 CCID₅₀. As defined, the vaccine is effective in general after a single administration via the oral and/or nasal route. However, repeated administrations may be necessary.

CLAIMS

1. Recombinant live vaccine comprising as vector a canine herpesvirus comprising and expressing at least one nucleotide sequence coding for a polypeptide, this sequence being inserted into a site which is non-essential for replication.
2. Recombinant live vaccine according to Claim 1, wherein the nucleotide sequence or sequences is/are inserted into at least one site selected from the group consisting of ORF3, ORF5, thymidine kinase gene and sequence situated between the CHV ORF19 and the CHV ORF22, by simple insertion or after total or partial deletion.
3. Recombinant live vaccine according to Claim 1 wherein, to express the inserted sequence, the vector comprises a strong eukaryotic promoter.
4. Recombinant live vaccine according to Claim 3, wherein the strong promoter is a CMV immediate-early promoter, preferably the murine or human CMV immediate-early promoter.
5. Recombinant live vaccine according to claim 1, which comprises at least two nucleotide sequences inserted into at least one site under the control of different eukaryotic promoters.
6. Recombinant live vaccine according to Claim 5, wherein the eukaryotic promoters are CMV immediate-early promoters of different animal origins.
7. Recombinant live vaccine according to Claim 5 which comprises a first nucleotide sequence associated with a CMV immediate-early promoter and another nucleotide sequence associated with another promoter, the two promoters in this case having their 5' ends adjacent.
8. Recombinant live vaccine according to claim 1, which comprises a nucleotide sequence coding for an antigenic polypeptide of a canine pathogenic agent, this sequence being inserted into one of the sites.

9. Recombinant live vaccine according to Claim 8, which comprises a sequence coding for an antigen selected from the group of antigens of the Carré's disease virus, of the rabies virus, of the canine parvovirus, of the canine parainfluenza virus, of the Rubarth's hepatitis virus, of *Bordetella bronchiseptica*, of *Borrelia burgdorferi*, of *Leptospira* spp. and of *Leishmania infantum*, this sequence being inserted into one of the sites.
10. Recombinant live vaccine according to Claim 9, which comprises a nucleotide sequence selected from the nucleotide sequences coding for the HA and F polypeptides of the Carré's disease virus.
11. Recombinant live vaccine according to Claim 9, which comprises at least one nucleotide sequence selected from the group of sequences corresponding to the rabies virus G gene, to the canine parvovirus VP2 gene, to the parainfluenza virus type 2 HA and F genes and to the *Borrelia burgdorferi* OspA and OspB genes.
12. Recombinant live vaccine according to claim 1, which comprises a nucleotide sequence coding for an immunomodulatory polypeptide, this sequence being inserted into one of the sites.
13. Recombinant live vaccine according to Claim 12, wherein this nucleotide sequence is selected from the group of sequences coding for cytokines.
14. Recombinant live vaccine according to claim 1, which comprises, inserted into the insertion locus, an expression cassette comprising in succession a promoter, two or more genes separated in pairs by an IRES, and a polyadenylation signal.
15. Polyvalent vaccine formula comprising, as a mixture or to be mixed, at least two recombinant live vaccines as defined in Claim 1, these vaccines comprising different inserted sequences.
16. DNA fragment consisting of all or part of the sequence defined by positions 583 to 4173 on the sequence SEQ ID No. 1.

XhoI

1 CTCGAGGAAATTGTTTGTGTTGTTATCTACAAAACCTTCAAAATATCTTTGTTTATTGTCCTCTTCGATGGATT
71 TTATTTTCATCTTCGCGATTGATTCTTCCTTGGTTACCGTAATTTATAAATAAACACAATAAAAAATTAAG
141 TTTAAAAACAATTTTATTAAACCCATCGTCTTGATTACTATCATCCCAGTAGGAAATTAGAACTAGATT
399 <...ValTrpArgArgSerLysSerAspAspTrpTyrSerIleLeuValLeuAsn
211 ATAATCTATCGGTATAGAAATATGTTTCCAAAATAAATTAGTTAAATTTTACGCTTTTCTTTATCATCT
381 <TyrAspIleProIleSerIleHisLysTrpPheLeuAsnThrLeuAsnLysAlaLysGluLysAspAspI
HindIII

281 ATAAAGCTTAAAGTGTTCATAAACAGATTATATCAAACTTTTCTTGATAATTGGAACCTTTTTTAA
357 <IlePheSerLeuLeuThrGluTyrValLeuAsnIleAspPheLysGluGlnIleIleProValArgLysIle
351 TTATAGATAAAATTTTACCCCTATATTCGGGTTATCATATTTGTTAGATGTTTAAATAAATTTTCTCTC
334 <IleSerLeuAsnGluGlyArgTyrGluProThrIleMetAsnThrLeuHisLysIlePheLysArgGlu
421 CAACACTTCGTGTTTGGTGTGGGGTGCCGAAGCATCATTAAAGAACGGGATATCGTTTTTCATTATTGGT
311 <LeuValGluHisLysThrGlnProAlaProLeuMetMetLeuSerArgSerIleThrLysMetIleProP
491 GGAAATCTTGATGTATATTTTAAATTTAACTATTCTCATCAACAGCTGTTACGCGCTTTGATTGTCCTT
287 <ProPheArgSerThrTyrLysLeuAsnLeuSerAsnGluAspValAlaThrValArgLysSerGlnGlyLys
561 TATTTGATGGAGAGTTTATTTTGGATAAAATTTTAAATCCATTTTGATTTTTTGGTATACCAAATGAATC
264 <AsnSerProSerAsnIleLysSerLeuIleLysPheGlyAsnGlnAsnLysProIleGlyPheSerAsp
631 GGTATCACTACTTTCCTACTCTGGTAATTTTGAGGATTCTTCGGATGATGAACTATATTTGTAGAAACA
241 <ThrAspSerSerGluSerSerThrIleAsnSerSerGluGluSerSerSerValIleAsnThrSerValS
701 GAATCACTTATTCTCCATGAGTTTGATATTTGATCTAAATATTTTTCATGATGTTGTATTTCTCCTGATT
217 <SerAspSerIleArgTrpSerAsnSerIleGlnAspLeuTyrLysGluHisHisGlnIleGluGlySerGln
771 CTTCAGATGAATCTCCACTATCAGAATTATATTCCTTTTACTATTTTATATTTTATTTTAAATAATTGA
194 <uGluSerSerAspGlySerAspSerAsnTyrGluLysLysSerAsnLysTyrLysAsnLysIleIleSer
841 TTGAACAGATTTTAAATAGGGGCTTGGTGCAAGTCTGTATGACAGCGAACAAACGTACATAAAACTCA
171 <GlnValSerLysLeuIleProAlaGlnHisLeuAspThrHisCysArgValPheThrCysLeuPheGluP
911 GGATATGATACATTTAAAGAAGCAAGTATATCCCTACATCGGAGGGTGGGTGGAAAAAGAGGTACAACAT
147 <roTyrSerValAsnLeuSerAlaLeuIleAspArgCysArgLeuThrProProPheLeuProValValAs
981 CCAATATAATATCACAAACCATTAATATTAGATCAGTATCCGTTGTATATATTTGAGCGGCTGTATTAGT
124 <pLeuIleIleAspCysGlyMetLeuIleLeuAspThrAspThrThrTyrIleGlnAlaAlaThrAsnThr
1051 ATGATAAAGATTAGCACAAACATCATCAGCTTCCATATCAGATACATTAAACATATGGAAAACCCAAATGG
101 <HisTyrLeuAsnAlaCysValAspAspAlaGluMetAspSerValAsnValTyrProPheGlyLeuHisA
1121 CGTATTAAATTAACACATAATTTATAACATACTTAGGAGTATTTACAAGTGAGCTCCATCGTGCTGATA
77 <rgIleLeuAsnValCysLeuLysTyrCysLeuLysProThrAsnValLeuSerSerTrpArgAlaSerLe
1191 AAATATTTTATAGGTTACATTTTTCCTTTTGTAAAGTTTAAATTTTCCCAACATACATTATCCTT
54 <uIleAsnIleProGluCysLysGluLeuLysLysTyrThrLysLeuIleGluGlyCysValAsnAspLys
1261 AATGGAATTTCTCCAAGTCTTCCAGATCTCTCTTGATGACACATAGTTTGTGTGGCGATACGTTTGCTCC
31 <IleSerAsnArgTrpThrLysTrpIleArgArgSerSerValTyrAsnThrHisArgTyrThrGlnGluV
<--- ORF1 BgIII

1331 ACGTTTAAACATGTCCATCACCATTATACCACGATCTGAAACAAAAATTGGAAAAATAAGATCTTTTTTGG
7 <alAsnLeuMetAspMetValMet

1401 AGTAATTTAAGTAAAGAAAAAAACATTACAGCTGTTACAGTGGGACTATCCGTTTGAGTATCATTTTCTA
1471 TACAAAATTTTCCATCAACGTATACATTACATTCCATAAGTCAATTGCGATTGGTGTACAATACCAGGT
SpeI

1541 GGTGTGCTATCGCATCGTGTTTAACTAGTCTACGACTATAAGCATATTTCAAAGTCCAAAAAGACCCA
SpeI

1611 TTTTAATAAAATACCAAACAGAACCTTTTCGACAACTAAATGAATAAACTAGTTTTTAAGTATTAAAT

FIG. 1

ORF2 --->

1681 ATAACCTTTAACTAAATTAATTAATAATGATTAATTTAAAAACCGAAATACAAATATTTTTTAGTCAAG
 1▶MetIleAsnLeuLysThrGluIleGlnIlePhePheSerGlnA
 1751 ATTTTATGAAATCAATCAAAATCACCACAATTATGCAAAATGAACCCACCTACCAACGTCATCAAAACTAA
 15▶spPheMetLysSerIleLysIleThrThrIleMetGlnMetAsnProProThrAsnValIleLysThrAs
 1821 TTTAGTCTATAAAAAGAAATTGTTAACATTTAGTTTAAATTTAAACTTTTATTTCTTAAAATTTTTATTA
 38▶nLeuValTyrLysLysLysLeuLeuThrPheSerLeuAsnLeuAsnPheTyrPheLeuLysPheLeuLeu
 1891 TTTTGCTTAGTTTTTAAGGCGATGGCGTGTTCCTGCTTAAACTGAATTTAAGATAACCAACCATCCAT
 62▶PheCysLeuValPheLysAlaMetAlaCysPheArgProLysThrGluPheLysIleThrAsnHisProS
 1961 CTCAGATTATAAATAACGAAGAAAATATAAACTCTGAAGAAGGAAAATTTATATCTGGTCGTGCTGTTTT
 85▶erGlnIleIleAsnAsnGluGluAsnIleAsnSerGluGluGlyLysPheIleSerGlyArgAlaValLe

HindIII

2031 GGAAGATCAAAAGCTTCGTGATGTGATAAGTATGCTAACACCCCTTTTCAACTAGCTTGAAAAACTCTTTT
 108▶uGluAspGlnLysLeuArgAspValIleSerMetLeuThrProPheSerThrSerLeuLysAsnSerPhe

SpeI

2101 ATAGTTTTTAGTGACTATGGGATGATGATCCATACTAGTATTTGTGGAGAACAATTTACATTCCTATTT
 132▶IleValPheSerAspTyrGlyMetMetIleHisThrSerIleCysGlyGluGlnIleTyrIleProIleS
 2171 CTAAAAACCAATTTTCTTCTTATTTTGGGGATATAGCGACCCCTGCGGTATTTTTGGCAAATGTTGATAG
 155▶erLysAsnGlnPheSerSerTyrPheTrpGlyTyrSerAspProAlaValPheLeuAlaAsnValAspSe
 2241 TAAAAGGGGATTGTTGGATGTTTTTAAATCAACAAGTAAATGTCTAAAGTATTCTTTGAAATAAGTAAC
 178▶rLysArgGlyLeuLeuAspValPheLysSerThrSerLysMetSerLysValPhePheGluIleSerAsn
 2311 CCTTCCCAACATAGAATGTTAAACAAGTTATTTTACTATAAGTGATAGTAATATAAAATGCTCTACAC
 202▶ProSerGlnHisArgMetLeuLysGlnValIlePheThrIleSerAspSerAsnIleLysCysSerThrL
 2381 TTCTAAAAGCTGAATTTAGTAAGTATTGTATTATGCTTCCATCAAGAAATCCGGACTTTAGTCTTGAAC
 225▶euLeuLysAlaGluPheSerAsnTyrCysIleMetLeuProSerArgAsnProAspPheSerLeuGluLe
 2451 TAATAAATATCAATTAATAAAATACTCGAACTAAACAAAAAACAATTCATTGTTAAAATTTGAATCT
 248▶uAsnLysTyrGlnLeuAsnLysIleLeuGluLeuAsnLysLysGlnAsnSerLeuLeuLysPheGluSer
 2521 AATGAAAATAATGTTGTGATTTTCATCTGAAAGTGAAGTGTTCATTGAATTTGGATAGAAGCGATTCTG
 272▶AsnGluAsnAsnValValIleSerSerGluSerGlySerValSerLeuAsnLeuAspArgSerAspSerG
 2591 AAGGAGAAGATAGCGCATCGATTTTAAATCTGCTACAAAAAAGTAAATCCTTATCTAGTTAAACACTC
 295▶luGlyGluAspSerAlaSerIleLeuLysSerAlaThrLysLysValAsnProTyrLeuValLysHisSe
 2661 AGAAAATTTCAAACGTTTAAATTTTCGTTGGATGATTATACCAATTTTTTTTCTCTTTTGAAAAAATA
 318▶rGluAsnPheLysArgLeuLysPheArgTrpMetIleIleProIlePhePheProLeuLeuLysLysLeu

HindIII

2731 AAATAACAAATACAACAGTATCGATAAATTTCTTTTTTACTCCAACCTACCAATCCCATGATAAGCTTGA
 342▶LysLeuThrAsnThrThrValSerIleAsnPhePhePheThrProThrThrAsnProMetIleSerLeuT
 2801 CGTCAAGTAAACCAATTGGAATTATACTGTTTTTCTTTTGTACCAATGAATTGCAACATAAGAGCCTGAA
 365▶hrSerSerLysProIleGlyIleIleLeuPhePhePheCysThrAsnGluLeuGlnHisLysSerLeuLy
 2871 GCGCCCAGCATCTCCATCAGATGAAGAAAAGCCACCAAAAATCCAATGTGGATTTTTTAGTCAACATTTT
 388▶sArgProAlaSerProSerAspGluGluLysProProLysIleGlnCysGlyPhePheSerGlnHisPhe
 2941 GTAAATACGGATGTTAATATTAAACCCTAATTAAATGACGTAAAATGATAAATTGTATTTAAAGAGAAGT
 412▶ValAsnThrAspValAsnIleLysPro...

HindIII

ORF3 --->

3011 TTTTTCAAAAGACAAGCTTTTATTAATAATGTCACCTAGAGAATAATAATGTACAATCGTTTGATCAACT
 1▶MetSerLeuGluAspAsnAsnValGlnSerPheAspGlnLe
 3081 GGAACCTCCTATTACATCATTTTCTATAATAAATGCTCTGGATCGAGACCTGGATGTCTACCATGTATG
 14▶uGluProProIleThrSerPheSerIleIleAsnCysSerGlySerArgProGlyCysLeuProCysMet

3151 TATGTAACACAAATCACTTCTATGTATTGGACTTCAAGCTGGAATTTTAACAGCCTTAATTATATTTAA
 38▶ TyrValThrThrLysSerLeuLeuCysIleGlyLeuGlnAlaGlyIleLeuThrAlaLeuIleIleLeuI
 3221 TTCAAATATTAACAGAAAGTTTCGTATGTTCTATAATTCCTATAGCAACTGTGTTAATATTTACGCTATC
 61▶ leGlnIleLeuThrGluSerPheValCysSerIleIleLeuIleAlaThrValLeuIlePheThrLeuSe
 3291 AAAAATATCTATTTCTACTTCTGAAAAAATTTCTTCTATTTGTAGAATTAGTCAGTCGATATTTGTAACA
 84▶ rLysIleSerIleSerThrSerGluLysIleSerSerIleCysArgIleSerGlnSerIlePheValThr
 3361 ATAGCCGCCTTTTGTGGGGGTTTGATGGATATTAAATCCAATAGCAATTAATAATCTTATATTTAA
 108▶ IleAlaAlaPheCysTrpGlyPheAspTrpIleLeuAsnProIleAlaIleLysIleIleLeuIleLeuS
 3431 GTTTATCATTTTAACTATTTGTACAATAAAAAATACATATATTTTATTTGATAAGTATATTAATGGTTC
 131▶ erLeuSerPheLeuThrIleCysThrIleLysIleHisIlePheTyrLeuIleSerIleLeuAsnGlySe
 3501 TGGATCTCATGTAAAAGGATCGCTAGTAACAATATTGTTTGGAACTATACTAGGTGTATTTGGAACCTCT
 154▶ rGlySerHisValLysGlySerLeuValThrIleLeuPheGlyThrIleLeuGlyValPheGlyThrLeu
 3571 AATGTTATTAATAAGAAATTTTAAATGGATTTGGTATAGCACTTTGTATAATTTTATCTAACACCAACT
 178▶ AsnValIleLysIleGluIleLeuIleGlyPheGlyIleAlaLeuCysIleIleLeuSerAsnThrAsnP
 SpeI BgIII
 3641 TTGGACTAGTAATTAGAGATACATGCTATTATCGTATAGGAAGATATAAATTAATGAGAAGCTTTTACAGA
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 3711 TCTTGGACATGGAGCGTCTTACTCAATAGAGGAAGATGAAACCTCTGATTACAGTGAAATACATGAAAGA
 224▶ pLeuGlyHisGlyAlaSerTyrSerIleGluGluAspGluThrSerAspTyrSerGluIleHisGluArg
 3781 AAAATTAGTAGTTTCAACTAATTTATAAATATCCAAGTATGATAATAATTTCTATTTTAGGATTTATGT
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 294▶ lIleSerPheIleValGlyHisCysLeuAlaPheLeuValGluProPheAsnTyrLysIleLysCysIle
 3991 TCACGAATTATACTAATTATTTGTCTTTTACTAGAATTAATTGCTTCACCTATTCTGTACTAGGATTAA
 318▶ SerArgIleIleLeuIleIleCysLeuLeuLeuGluLeuIleAlaSerLeuIleSerValLeuGlyLeuA
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 ORF4 --->
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 1▶ MetSerPheLysAsnPheTyrLeuIleTyrValIleI
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 60▶ LeuLysSerValSerThrSerIleLysProProLysAsnLeuLysLysLysLeuLeuLysSerLysCysA
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 4761 GAAAGTTTAAAAACAGATATATCAAAAAATATTTTATTATTTTCGACAAAAAATAATAGTGATACTTTT
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 5671 TAATTGGTGGAGCCATTTTGTATAGTATTGATTTTATAACAGCTTTATGTTTTATTGTTCAAAAAA
 433 ▶ leIleGlyGlyAlaIlePheValIleValLeuIlePheIleThrAlaLeuCysPheTyrCysSerLysAs
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 456 ▶ nAsnLysIle...
 ORF5 --->
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 1 ▶ MetAlaGln
 5881 TTAGTTTTAACTGATATTCCCCTCGAAGATGTGGAAAATAAAAACTTCATCCGACGAAGAAACAATA
 4 ▶ LeuValLeuThrAspIleProLeuGluAspValGluAsnLysAsnThrSerSerAspGluGluThrThrA
 5951 ACTTAAACCAGAAAAATCAACATGTCAATGTTTATGTGTTACCCTTGGATTTTTTGCAGCTGGAATTTT
 27 ▶ snLeuAsnGlnLysLysSerThrCysGlnCysLeuCysValThrLeuGlyPhePheAlaAlaGlyIleLe
 6021 ATTAACCATAGCTGCAATAATTTTACTTTTATTTTACAGTACCATTAGAAATGCTTGGATCGATTAAT
 50 ▶ uLeuThrIleAlaAlaIleIlePheThrPheIlePheThrValProLeuGluMetLeuGlySerIleAsn
 6091 TGTCTCCATCTACATTGCTATTGATAATGTTTGTATCGAACCAATAAAAAATCTATTAATCTTATT
 74 ▶ CysProProSerThrPheGlyIleAspAsnValCysIleGluProIleLysLysSerIleAsnSerTyrS
 Scal
 6161 CAGAATTATCTAAAATATGTTATGATAGATTGTCAAATCCGATAAATCAGAGTACT
 97 ▶ erGluLeuSerLysIleCysTyrAspArgLeuSerAsnProIleAsnGlnSerThr

(FIG. 1 end)

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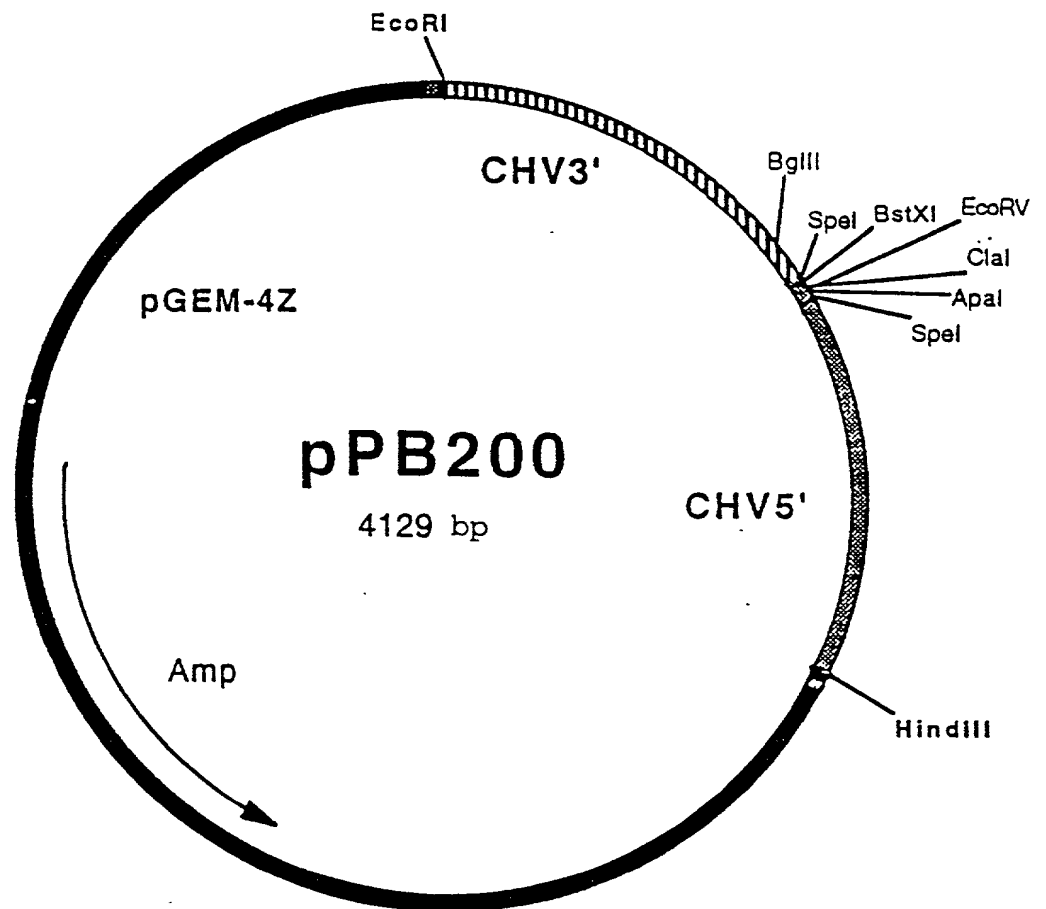


FIG. 2

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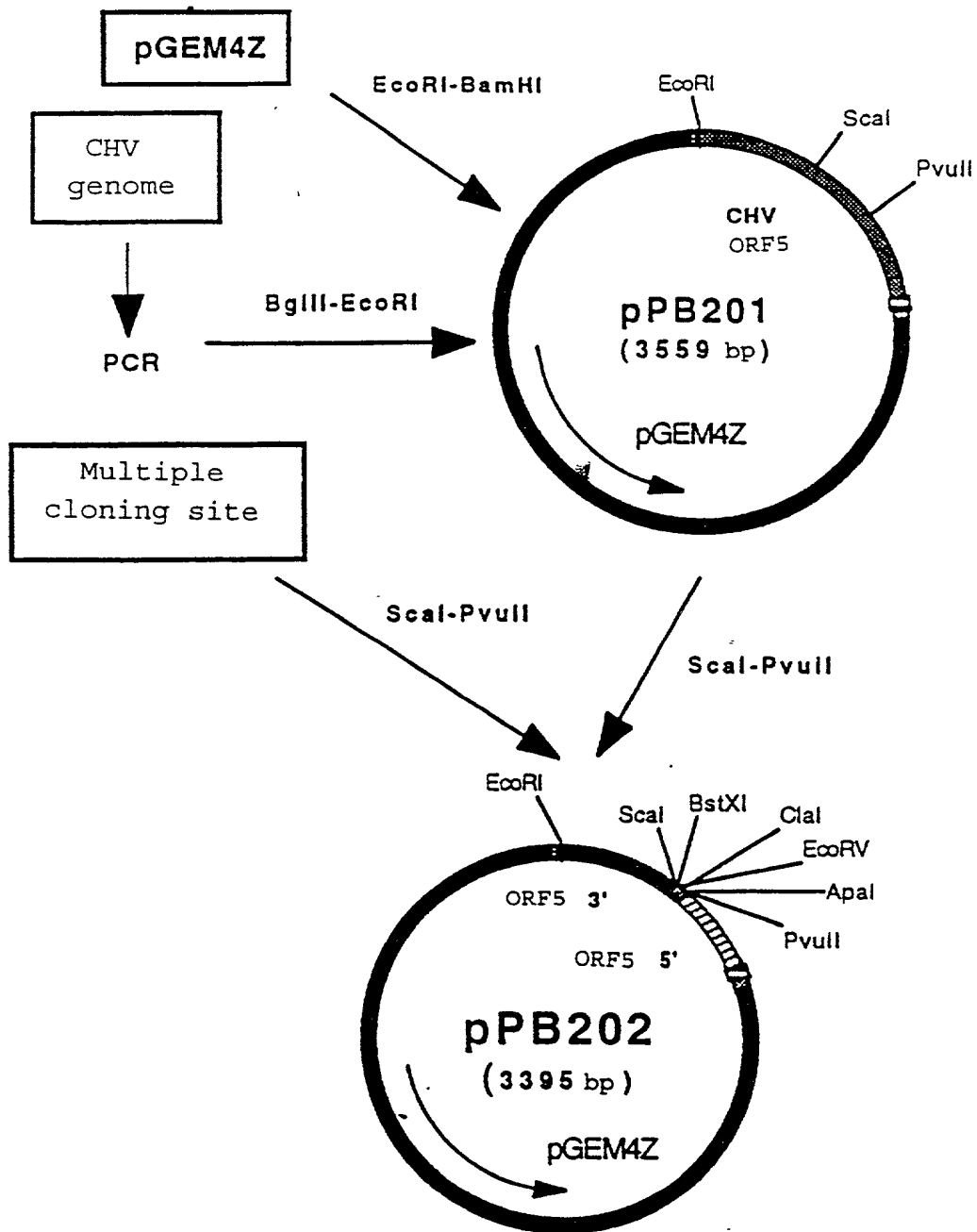


FIG. 3

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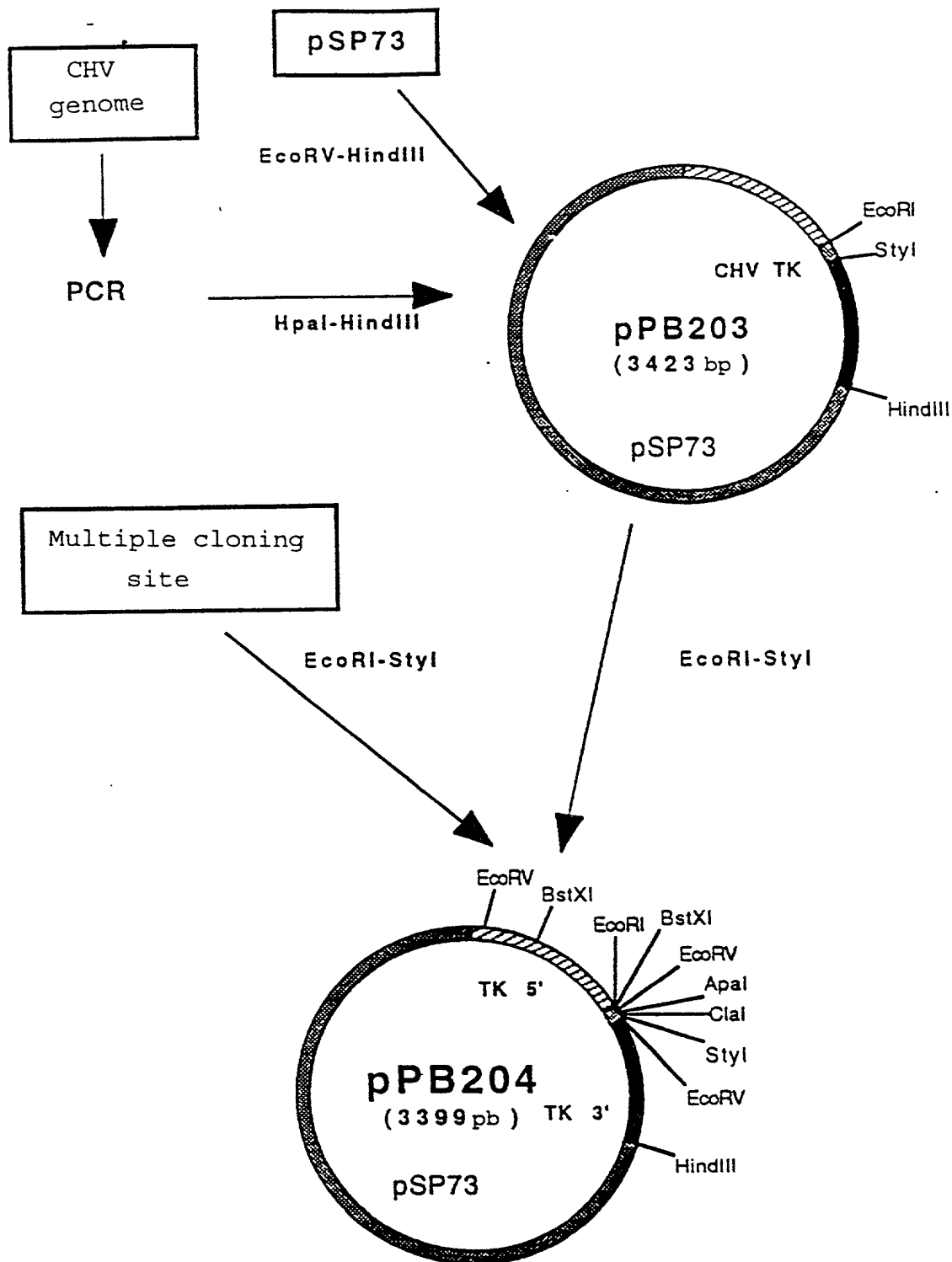


FIG. 4

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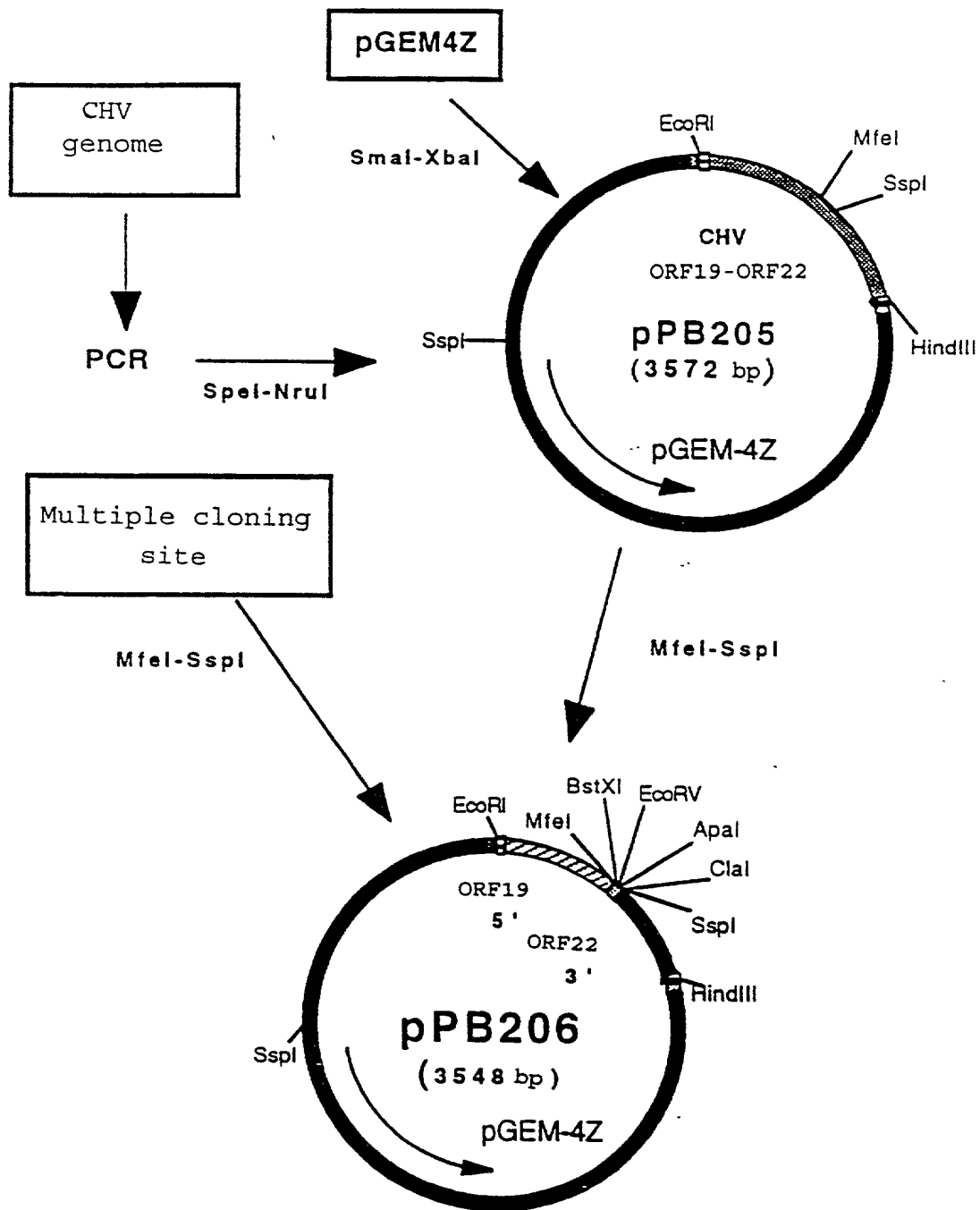


FIG. 5

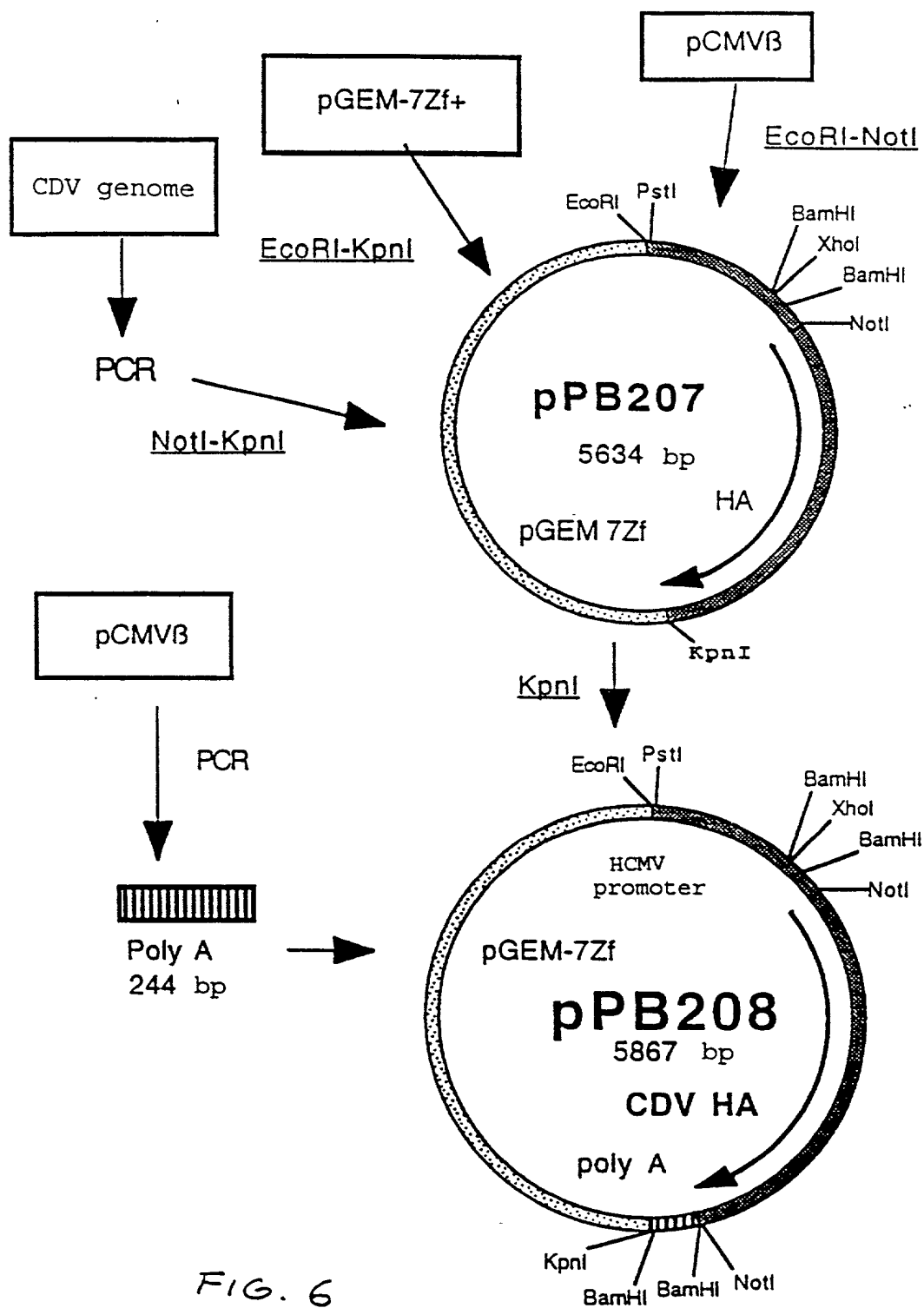


FIG. 6

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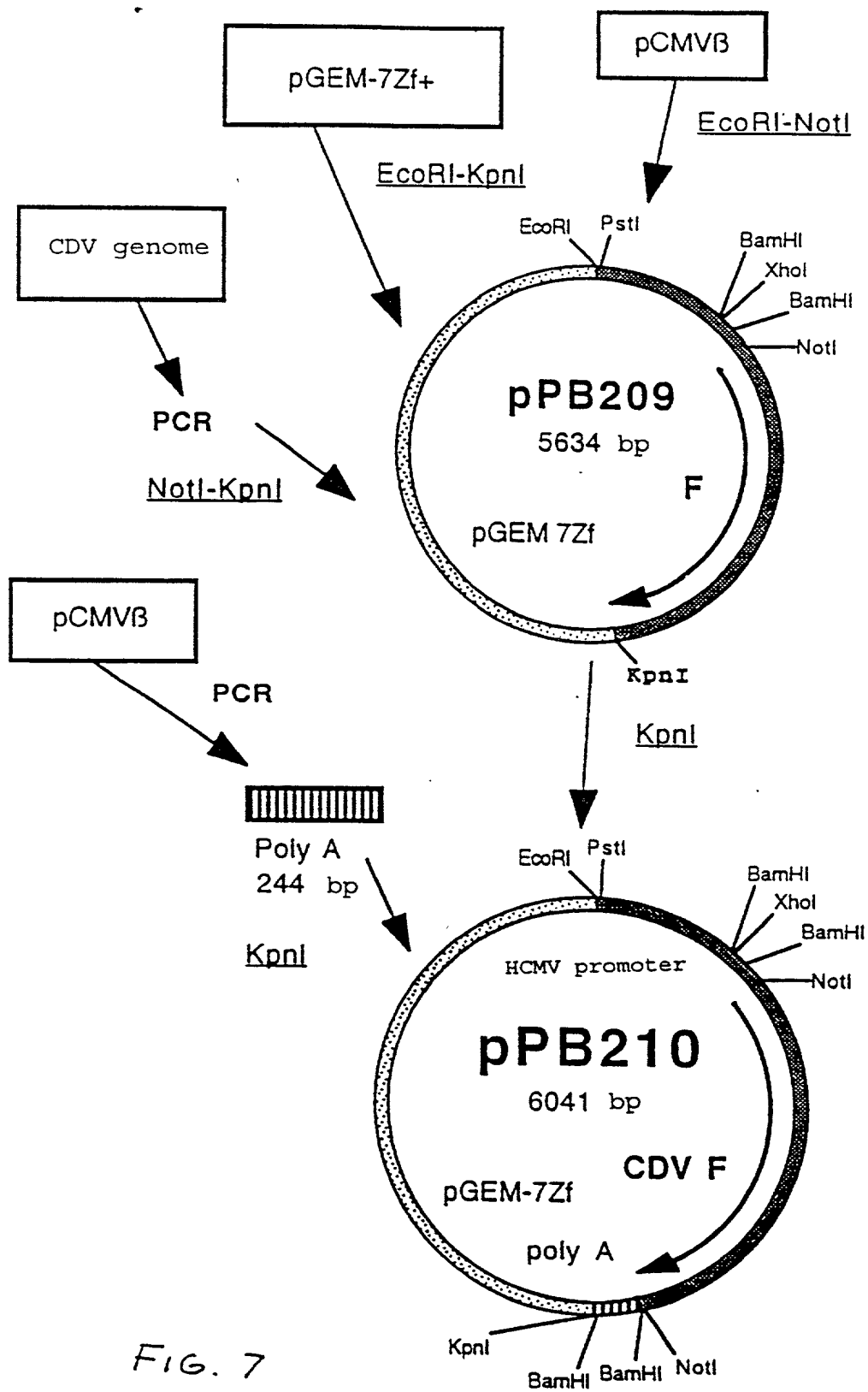


FIG. 7

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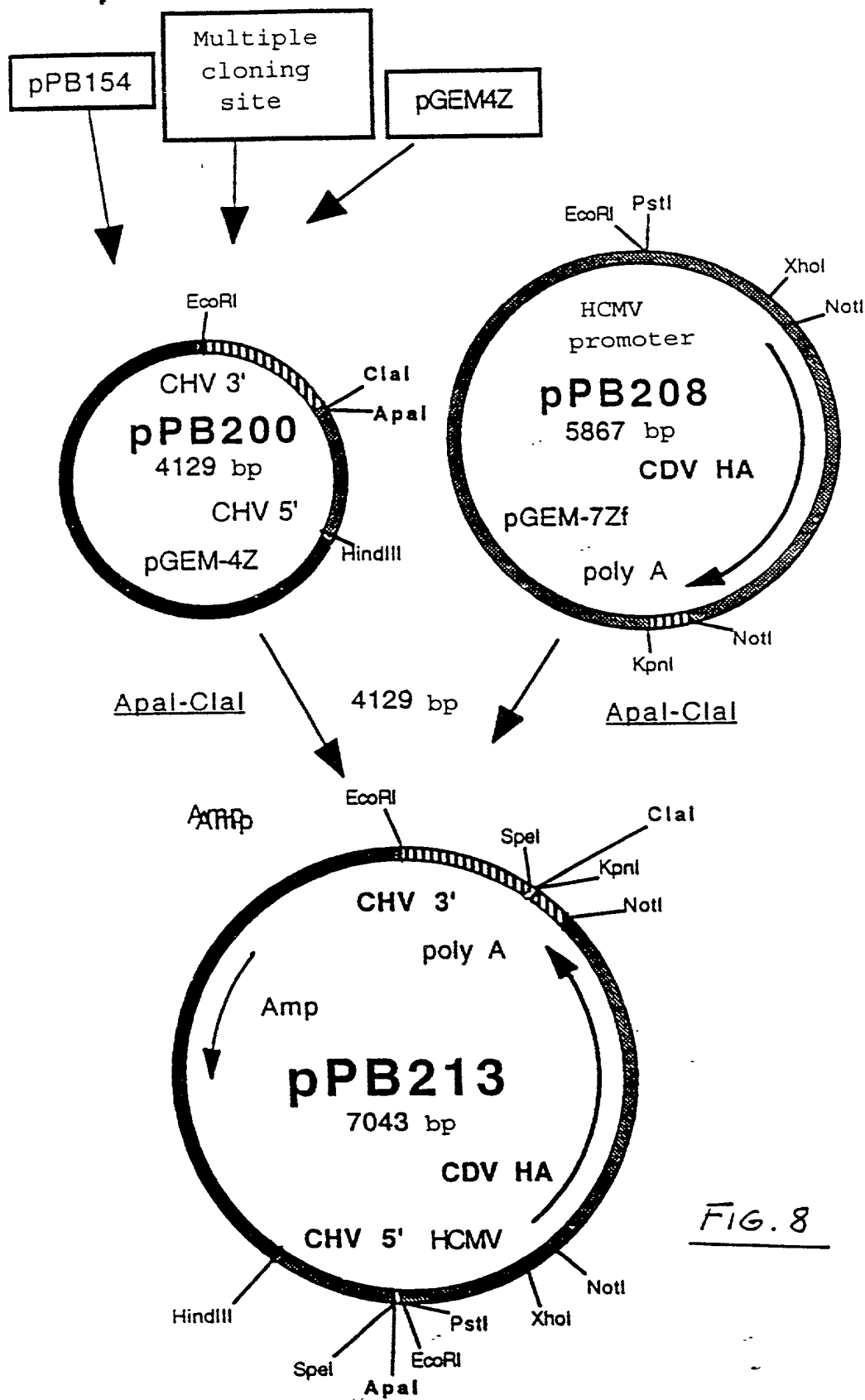


FIG. 8

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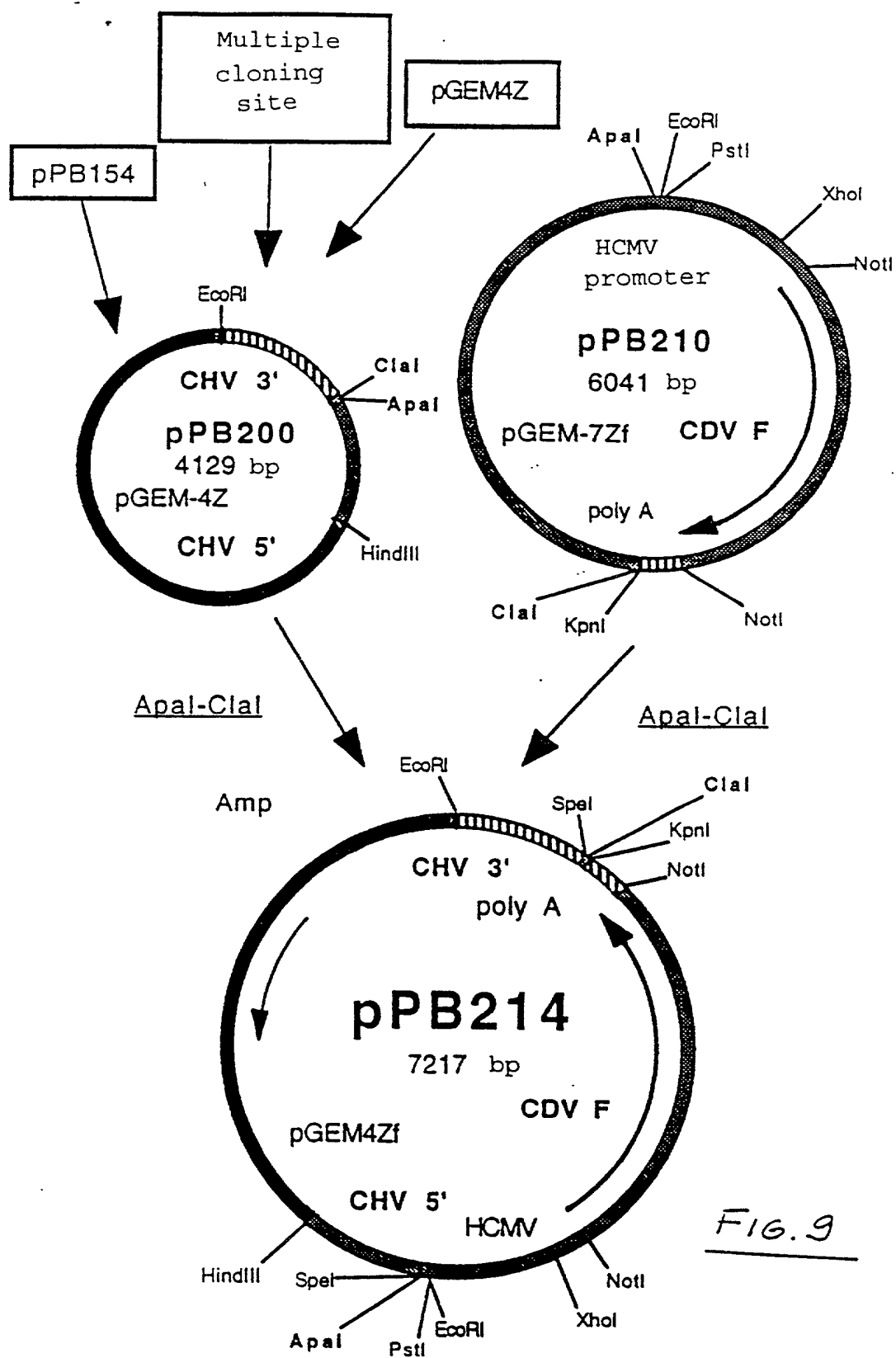


FIG. 9

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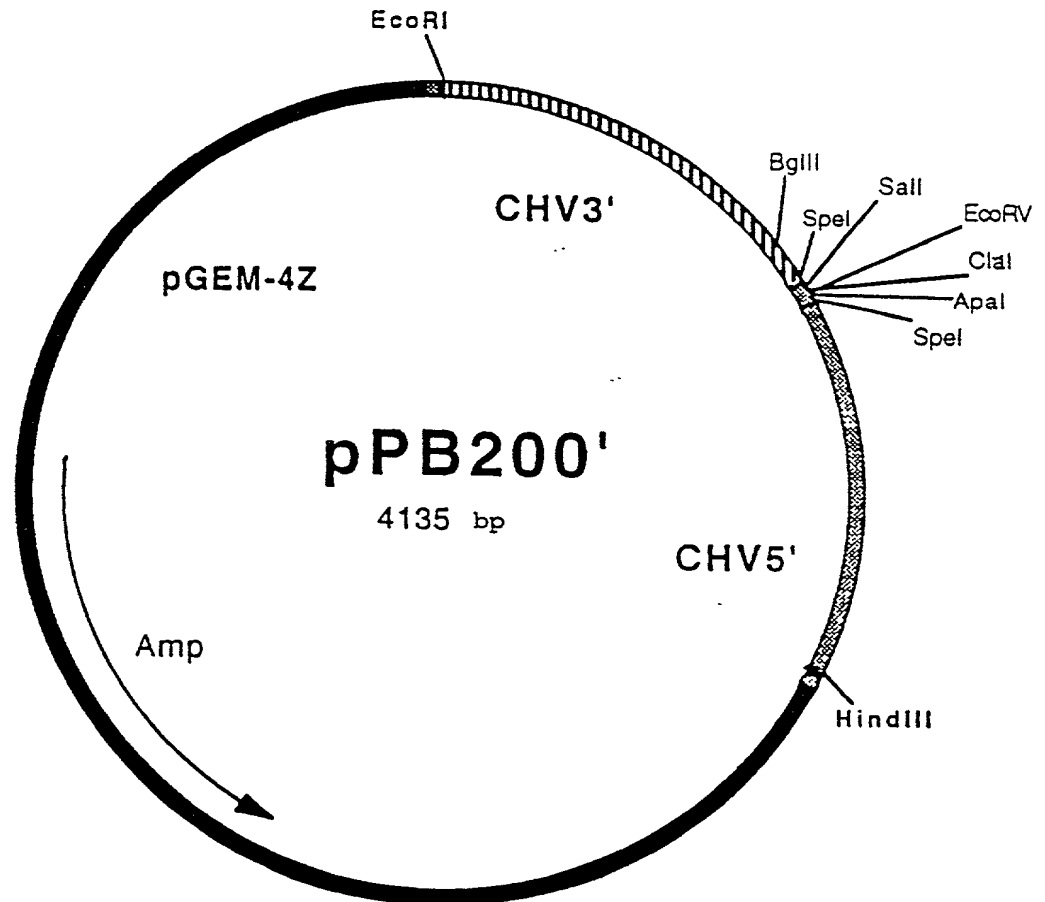


FIG. 10

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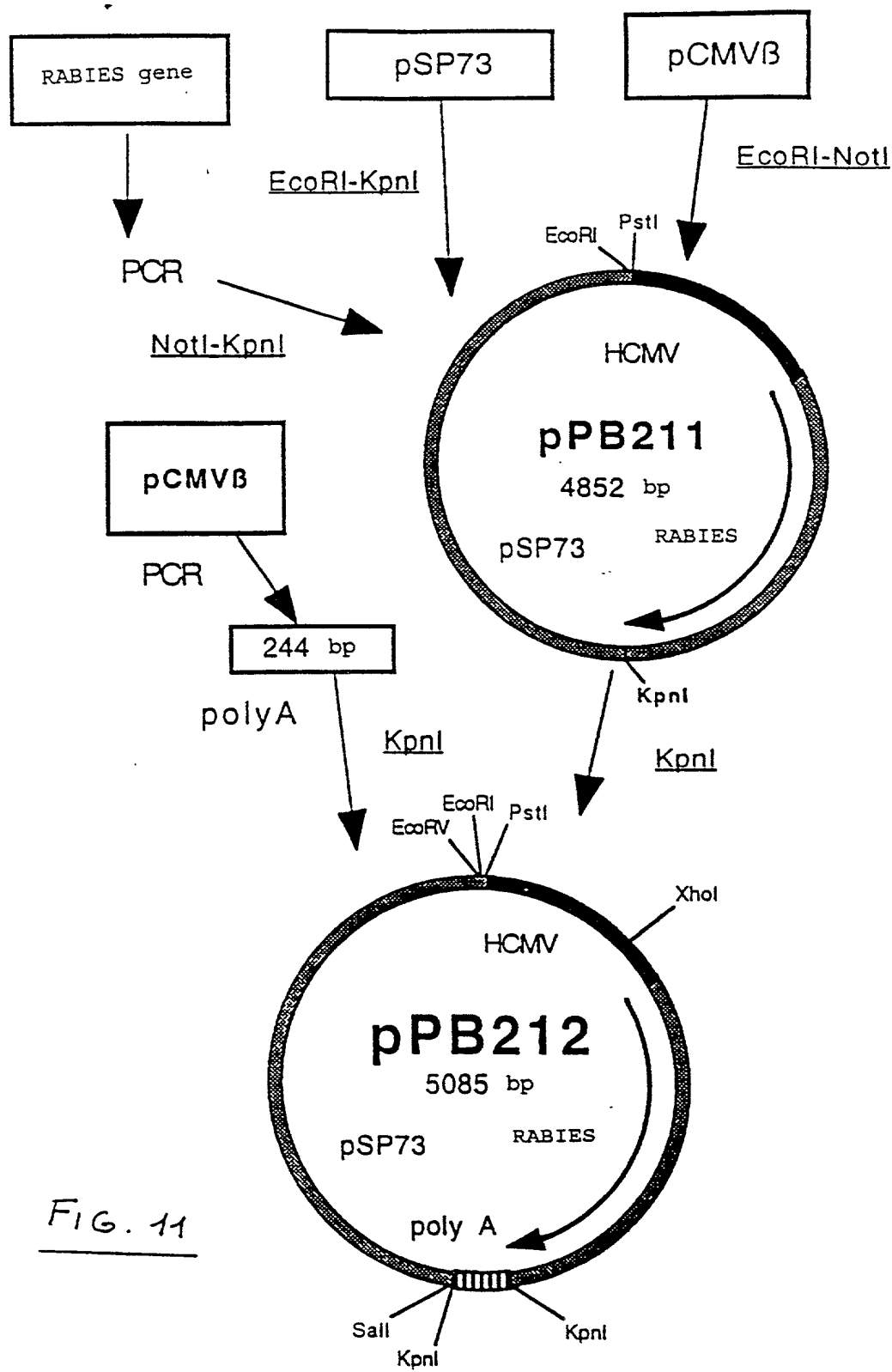


FIG. 11

000190-62495500

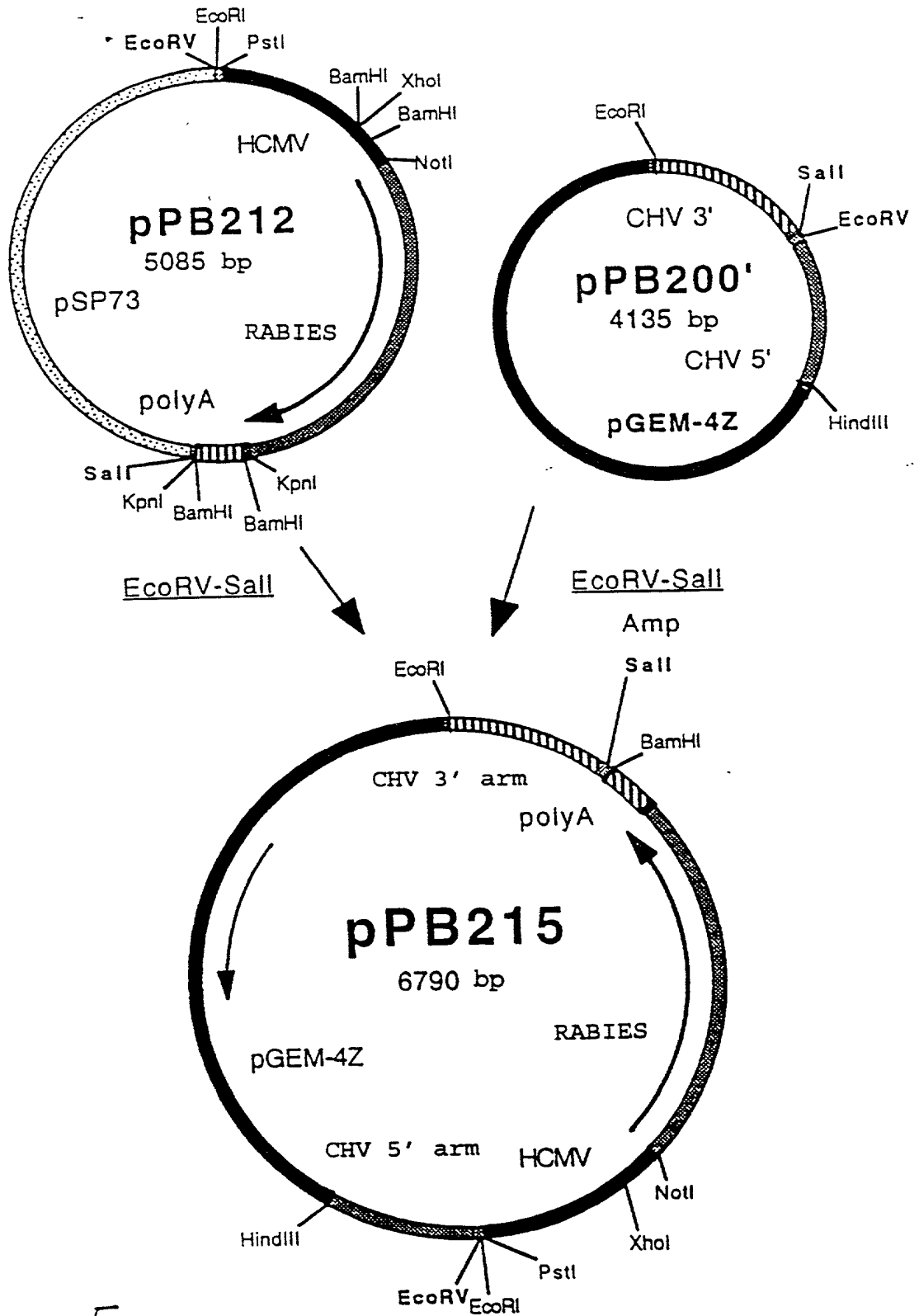


FIG. 12

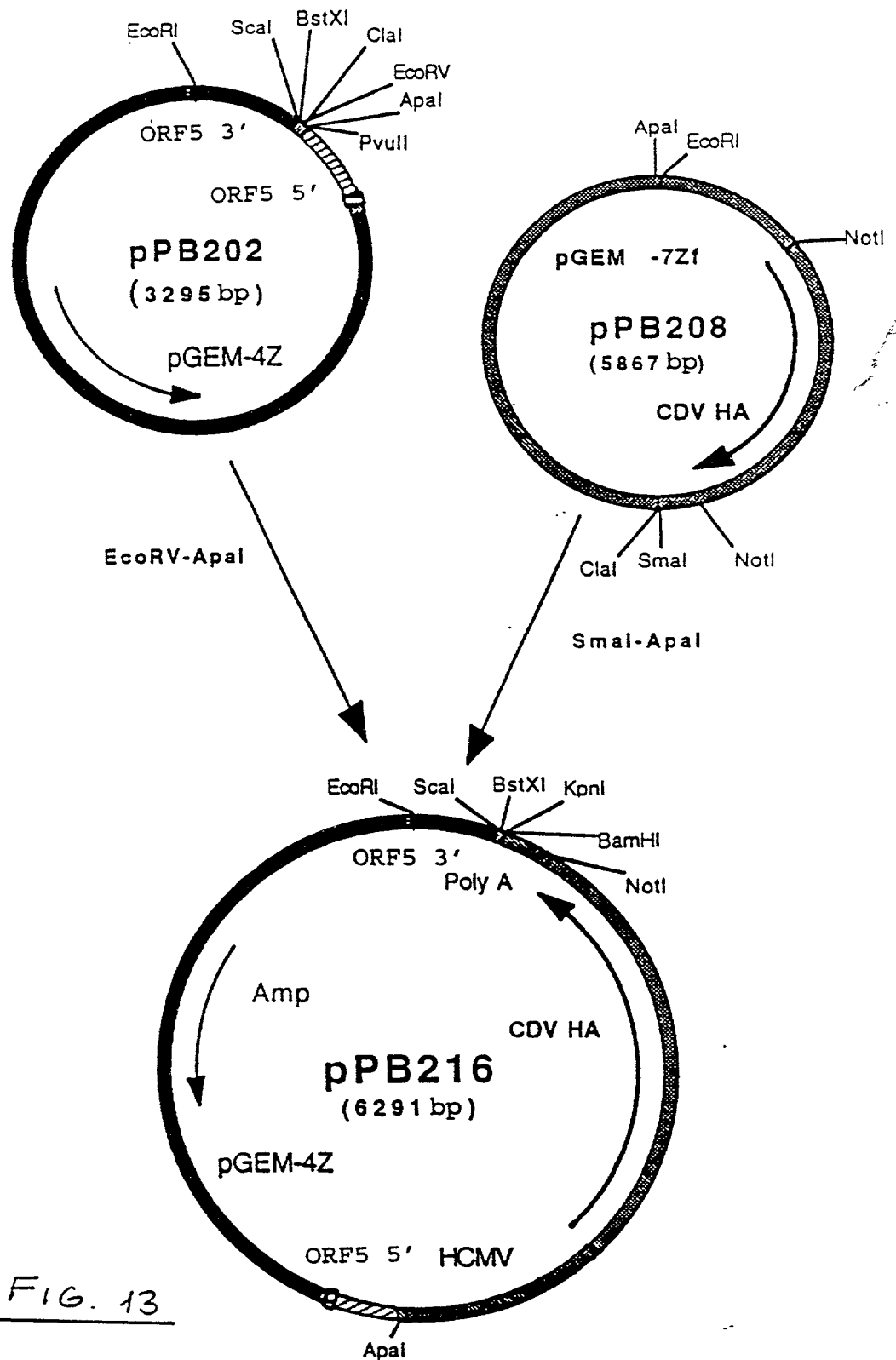


FIG. 13

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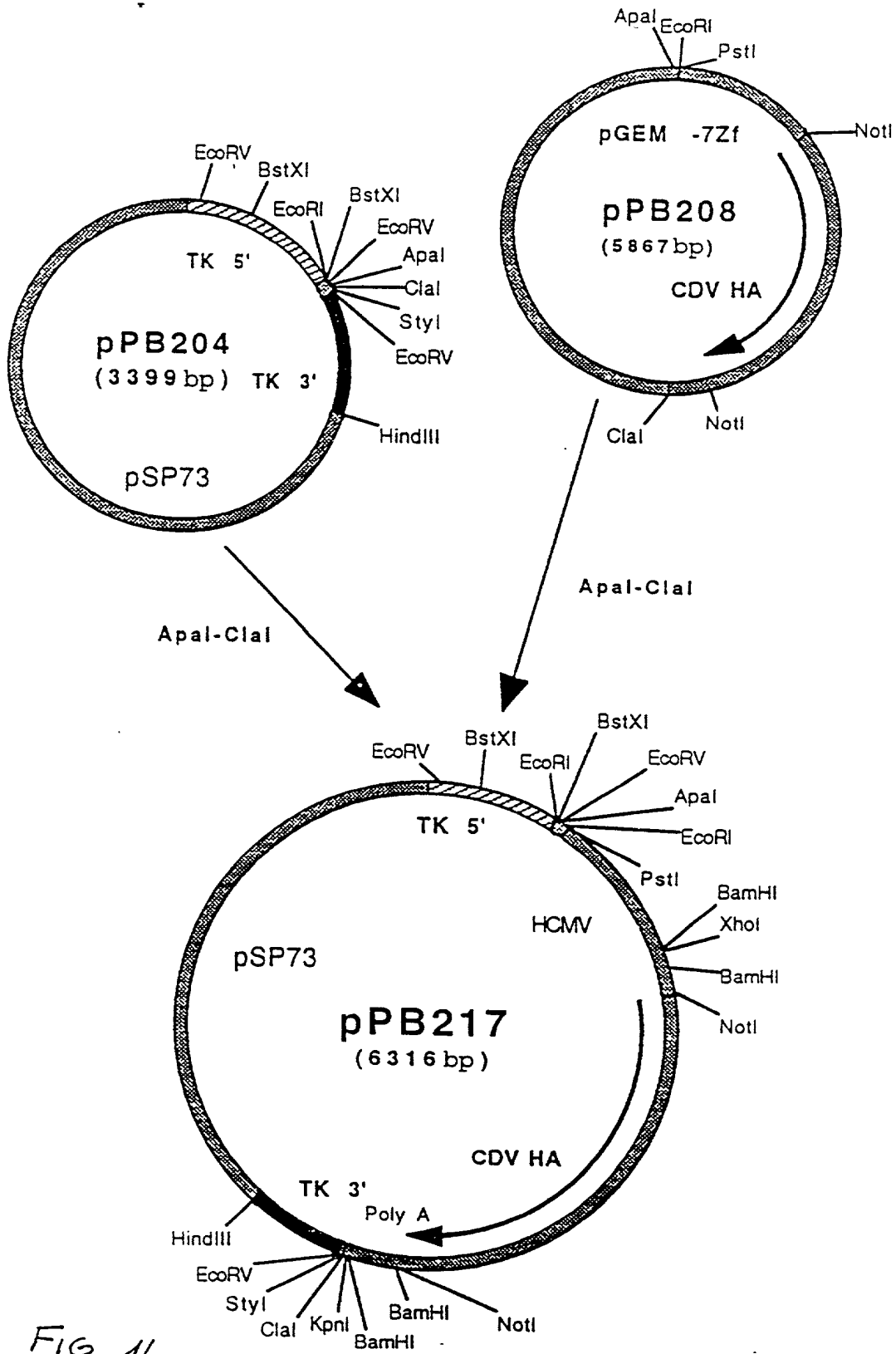
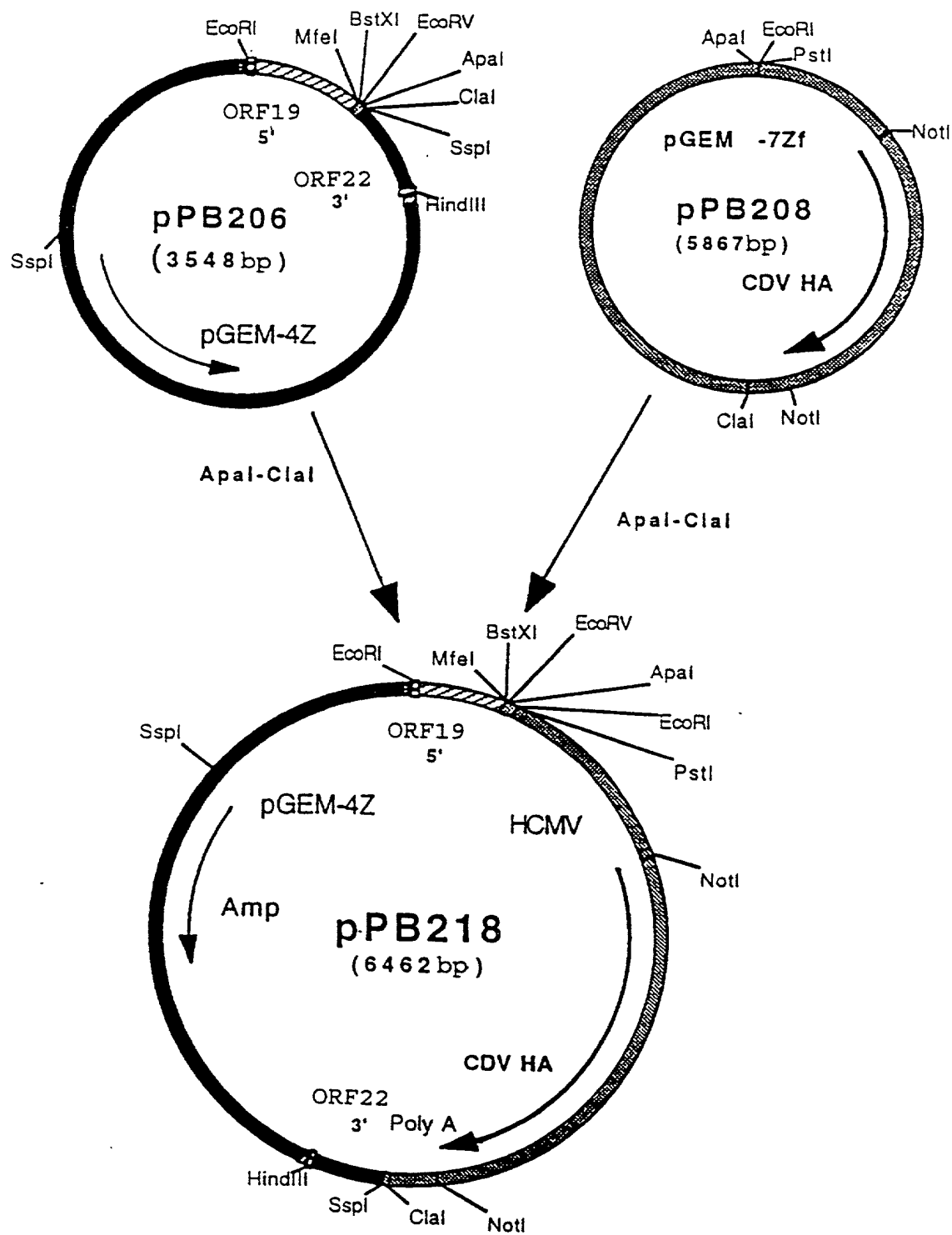


FIG. 14

FIG. 15

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Under 37 CFR § 1.63; includes reference to PCT International Applications)

FROMMER LAWRENCE & HAUG LLP
File No.: 454313-2200

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED: **CANINE HERPESVIRUS BASED RECOMBINANT LIVE VACCINE, IN PARTICULAR AGAINST CANINE DISTEMPER, RABIES OR THE PARAINFLUENZA 2 VIRUS**, the specification of which

is attached hereto

x was filed on December 16, 1998 as United States Patent Application Serial No. 09/213,053, with amendments through even date herewith (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

<u>Country (or PCT)</u>	<u>Application Number:</u>	<u>Filed (Day/Month/Year)</u>	<u>Priority Claimed:</u>	
			<u>Yes</u>	<u>No</u>
FRANCE	96/08242	27 June 1996	x	
PCT	PCT/FR97/01115	23 June 1997	x	

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. PCT Application(s) [list additional applications on separate page]:


<u>PCT Appln. No.:</u>	<u>Filed (Day/Month/Year)</u>	<u>Status (patented, pending, abandoned)</u>
PCT/FR97/01115	23 June 1997	Pending

I hereby appoint WILLIAM S. FROMMER, Registration No. 25,506 and FROMMER LAWRENCE & HAUG LLP, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional

In testimony of which I have affixed our signatures.


February 1st, 1999

Date _____


Jean-Christophe AUDONNET

February 1st, 1999

Date _____


Philippe BAUDU